Infra-red analysis and the intermolecular interactions of ingredients during baking: enzymatic hydrolysis of starch, protein secondary structure and product characteristics

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

MOHAMED AHMED OMER

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Publications

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Abstract

Wheat flour doughs are important in the manufacture of various bakery products. There is a complex array of intermolecular interactions occurring within doughs and during the baking process; these interactions determine the end product quality and consumer appeal. In this study the inter-molecular interactions of flour components and other baking ingredients were investigated in the main three stages of bread making. These are, firstly, the mixing stage which primarily involves protein components, then the fermentation process which depends on both protein and starch because this requires both the production and retention of gas. Finally the baking process impacts end product quality through the changes of staling following the interactions occurring in the earlier stages.

The primary aim of this study has been to investigate the inter-molecular interactions of selected baking ingredients during the three main stages of bread making process. The specific objectives have been to monitor the impact of the interactions at each stage as well as the impact on the ultimate characteristics of the resultant bread. In seeking to provide chemical and biochemical interpretation for the ingredient interactions during the dough mixing and proofing stages as well as their impact on the final product quality, an approach based on Attenuated total reflectance Fourier transform infrared (ATR/FT-IR) spectroscopy has also been evaluated. The purpose has been to seek new ways to investigate the complexity of these systems and adapting modern instrumental techniques to the study of wheat flour doughs.

In this study ATR/FT-IR spectroscopy has been used and adapted to an investigation of the interactions occurring during bread making. Recent innovations and software which facilitate deconvolution of spectral data have been applied. In addition, baking tests were used to study the impact of ingredient interactions on the quality attributes of the end product. The paramters evaluated included texture and bread staling measured using texture analysis, bread colour using Minolta Chroma Meter as well as bread volume.

The interaction of baking ingredients during the dough development stage were investigated using ATR/FT-IR spectroscopy particularly utilising its capability of showing the relative contributions of the different types of protein secondary structure, specifically α-helix, β-pleated sheet and β-turns. The results obtained during this study showed that the wheat flour proteins in the dough are the primary determinant of the dough development process. The changes taking place in the dough structure have been interpreted in relation to the changes in the protein secondary structural components. The relative proportion of the β-sheet content increased upon addition of the oxidising agent

ascorbic acid. The resultant changes in dough strength reflect the increased contribution of the β-sheet structure. Since the glutenin components of the gluten matrix are known to be rich in β -sheet, the dough strength has been interpreted in relation to the role and involvement of the glutenin within the matrix. Conversely, the incorporation of the reducing agent L-cysteine weakens the dough structure. This appears to be reflected in an increase in the intra-molecular interactions, protein- protein as well as starch- protein. The results are also interpreted as indicating that the oxidising agent increases inter-molecular interactions within the same protein molecule by enhancing the formation of disulfide bonds.

A further aspect of molecular interaction investigated during the fermentation process was the action of α-amylase on wheat flour starch. This was monitored and measured, using ATR/FT-IR in the region of 800-1300 cm^{-1} corresponding with vibration associated with C-C and C-O bonds. In order to study the inter-molecular interactions during the fermentation process, time dependent measurements were taken to monitor the reaction in which α-amylase hydrolysed starch components of wheat flour. In the spectra, peaks corresponding to the various sugars were detected, the relative areas of each were calculated and the amount of the sugars was observed to increase during the reaction. The rate of cleavage of the glycosidic linkage in starch by the action of α-amylase was monitored over the incubation time. The changes in starch structure were also estimated during this study focussing on the peak at 1045 cm^{-1} which indicates the crystallinity of the starch. This was seen to decrease while increases were seen in the peak at around 1022 cm^{-1}, corresponding with the amorphous structure of starch. In addition, it was possible to quantitate the peak appearing at approximately 976 cm^{-1} and this is identified as the damaged starch component.

The interaction of ingredients during baking and their impact on the end product quality were investigated. The addition of various levels of the oxidising agent ascorbic acid increased bread volume and enhanced bread texture while the reducing agent L-cysteine increased bread volume to certain extent, but with elevated levels of incorporation, marked decreases in volume occurred, whilst at all levels bread crust color was improved. These changes in the baking quality were related to the changes brought about by the same dosage of both ingredients in the previous two steps of dough development and the fermentation stage. The effects of various levels of α-amylase on bread quality were measured and the addition of α-amylase was found to enhance bread volume, texture and colour as well as slowing the rate of bread staling.

The current study enhances our knowledge of the baking process, confirms the value of deconvolution software for the evaluation of infra-red spectra as well as demonstrating the value of infra-red analysis as a means of monitoring the changes taking place and the influence of improver ingredients.

Abbreviations

ATR	attenuated total refelectance	WS-AX	water souluble arabino xylan
ASA	ascorbic acid	WU-AX	water unsouluble arabino xylan
α	alpha	S-AX	soluble arabino xylan
β	beta	WE-AX	water extractable arabino xylan
γ	gamma	WEP	water extractable pentosan
ω	omega	RACI	Royal Australian Chemical Institute
DATEM	diacetyl tartaric acid esters of monodiglycerides	ml	millilitre
SSL	sodium stearoyl-2-lactylate	ppm	parts per million
CSL	calcium stearoyl-2-lactylate	µm	micrometer
FT-IR	Fourier transform infrared	λ	lambda
IRE	internal reflection element	θ	theta
KBr	potasium bromide	%	percentage
D_2O	deuterium oxide	DHA	dehydro ascorbic acid
HMW	high molecular weight	AOX	ascorbate oxidase
LMW	low molecular weight	SKB	Sandstedt - Kneen - Blish
EC	Enzyme Commission	U.S.	United states
BU	Brabender units	ºC	degrees Celsius
CM	centimeter	NMR	nuclear magnetic resonance
N	nitrogen	SH	sulfdryl
FT	Fourier transform	S-S	disulfide
OH	nydroxyl	IUBMB	International Union of Biochemistry and Molecular Biology
min	minutes	ATP	adenosine triphosphate
BRIA	Bread Research Institute of Australia	NAD	nicotinamide adenine dinucleotide
g	gram	NADP	nicotinamide adenine dinucleotide phosphate
kg	kilogram	EC	Enzymes commission
AACC	American Association of Cereal chemists	GSH-DH	glutathione dehydrogenase
s	seconds		

T	*Triticum*
mm	millimeter
CFV	compression force value
N	Newton

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1 Introduction

1.1 Stages of bread making

During the baking process various ingredients are used to enhance the baking performance of different types of flour with differing chemical composition. These additives have diverse chemical structure and include enzymes, oxidising and reducing agents, as well as emulsufiers. Recently enzymes have increasingly been preferred by the baking industry as they are natural. Understanding the chemical and physical changes during the various stages of bread making, is the key factor in controlling the action of baking ingredients throughout the various steps in the baking process. [1, 2] Bread dough systems are multiphase and have many components, with the major ones being starch, protein, lipids, other carbohydrates, water, and air. The quality parameters of bread are appearance, texture, taste, and stability and these are determined by the macroscopic structure of the product. This, in turn, is greatly influenced by dough ingredients and processing conditions. The macroscopic structure is developed during the baking process through mixing and kneading of the ingredients, proving the dough and baking. [3]

1.1.1 Dough development stage (Mixing)

Dough mixing is a very important stage in bread making. It performs three primary functions. Firstly, hydration occurs as the ingredients are homogenised and evenly distributed into the flour which is the main ingredient. On a weight for weight basis, most of the water added to make up the dough is absorbed by hydrophilic groups on the protein molecules.[4] The second function is the mechanical development of the gluten network, necessary to trap and hold the gases produced during the fermentation stage. The third function is dough aeration and, during the initial mixing stage, the gas bubbles in the dough are first created.[5, 6] In fact a significant component of the dough is air, which

arises from air entrapped in the bulk volume of the flour mass or from entrainment during the mixing process.[4, 7] The contribution of this air in the baking process is lowering the density of the dough, as shown in Figure 1-1, supplying oxygen for the yeast growth, and creating the gas bubbles which act as nuclei for homogenous expansion. [7]

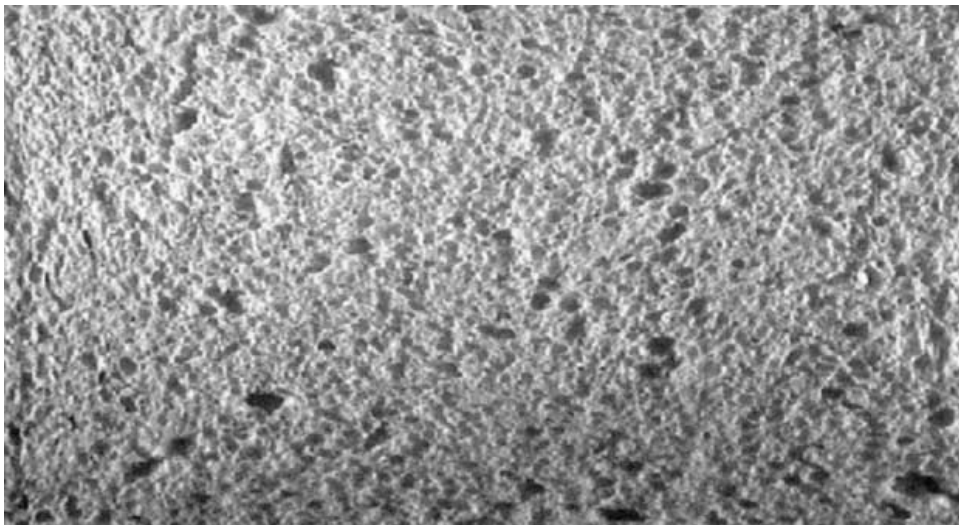

Figure 1-1 Digital image of bread crumb showing the effect of mixing in the grain of the crumb, adapted from [4]

Dough can be regarded as a foam and this is formed during the mixing process. A continuous protein network is formed, air bubbles are created and larger bubbles are broken up into smaller bubbles. [8] Dough mixing characteristics, rheological properties and bread quality are very much related to protein composition. [9, 10] The extent to which dough should be mixed and developed depends on the type of flour and its chemical composition, the bread making method used and the subsequent processing Steps as shown in, as well as the desired end product characteristics. [11]

The amount of mixing required to achieve optimum dough development may be determined through a variety of means. The first approach is to perform baking trials on dough mixed for different lengths of time possibly with differing mixing speeds. For the Chorleywood bread processes a minimum mechanical energy input of 40kJ kg^{-1} with no more than 5min mixing time is recommend. [6] During mixing, gliadin and glutenin form disulfide bridges producing the gluten protein matrix which is a three–dimensional structure capable of stretching without breaking as shown in Figure 1-2.

Table 1-1 Mixing time for different bread making methods for North American wheat. [11]

Process	Total mixing time (min)	Total fermentation time (h)	Cysteine added (ppm)
Sponge and dough	15-17	3-5	-
Straight dough	16-21	2	-
No-time straight dough	16-21	-	40

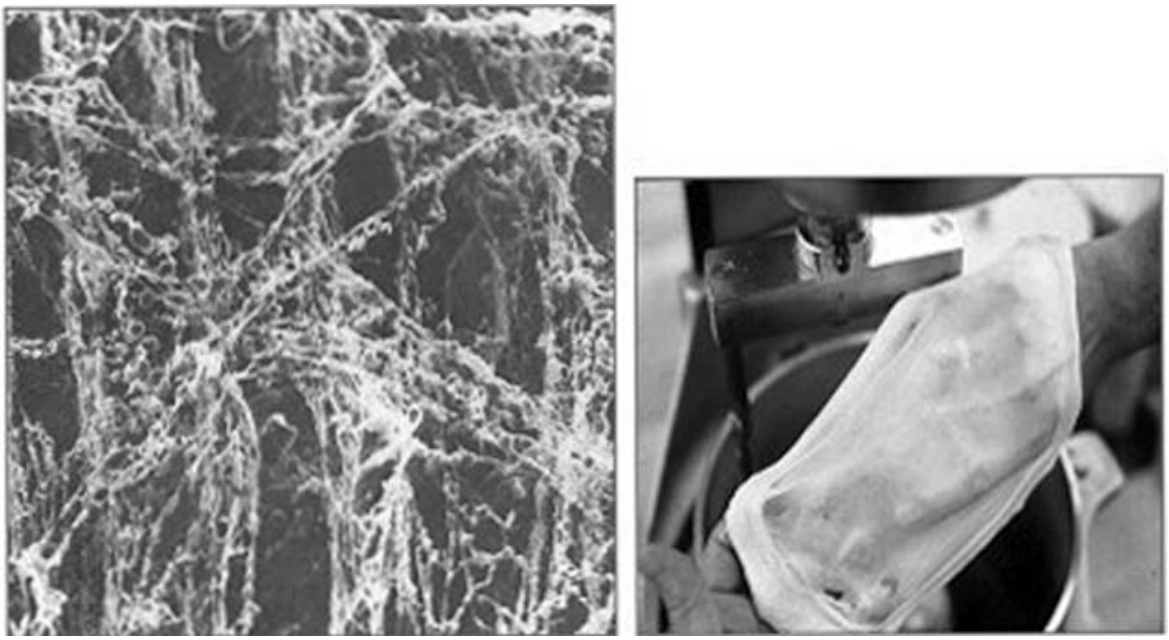

Figure 1-2 Gluten network development and gluten extension adapted from [12]

The three dimensional structure of the gluten may break with overextension if the dough is kneaded too much or over mixed. Therefore mixing time is an important factor in determining the end product quality. [10, 12] Mixing energy on the other hand plays an important role in optimising bread quality and final bread volume during the mechanical dough development as shown in Figure 1-3. [13] The high energy input facilitates the breaking of disulfide bonds which hold the original protein configuration together. As a

result, the sites available for the oxidation will increase. In addition, the energy input during mixing causes a considerable temperature rise in the final dough. [13, 14]

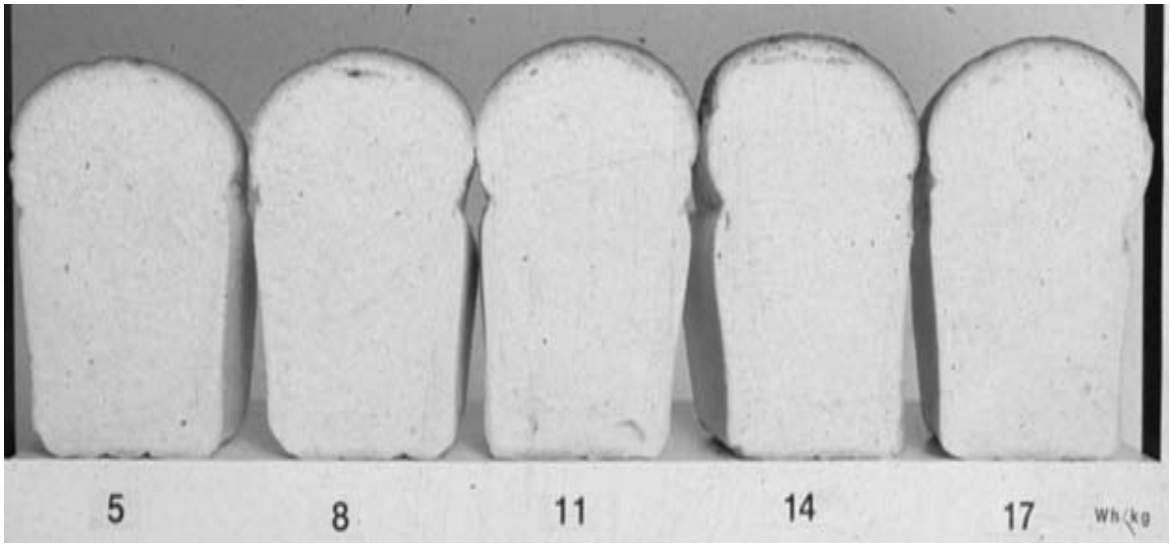

Figure 1-3 The effect of energy input (Wh/kg) on bread volume. [13]

1.1.2 Dough proving

Proving is the heart of the bread making process; it is the very important link between mixing and baking.[15] Understanding how aeration during mixing affects bread quality requires a knowledge therefore of how the bubbles in the dough grow and change during proving. [16] Bubble growth during proving is influenced by four factors: the first is the rate of carbon dioxide production, which is a function of the rate of yeast growth and multiplication. Yeast multiply by budding as shown in Figure 1-4. [17]. Carbon dioxide is one of the by products of yeast growth and another condition that favours yeast growth is the availability of food in the form of fermentable sugars. This is typically provided by the addition of α-amylase to the bread formula to work upon damaged starch to produce fermentable sugars and can also be aided by the addition of sucrose to the bread formula. [16]

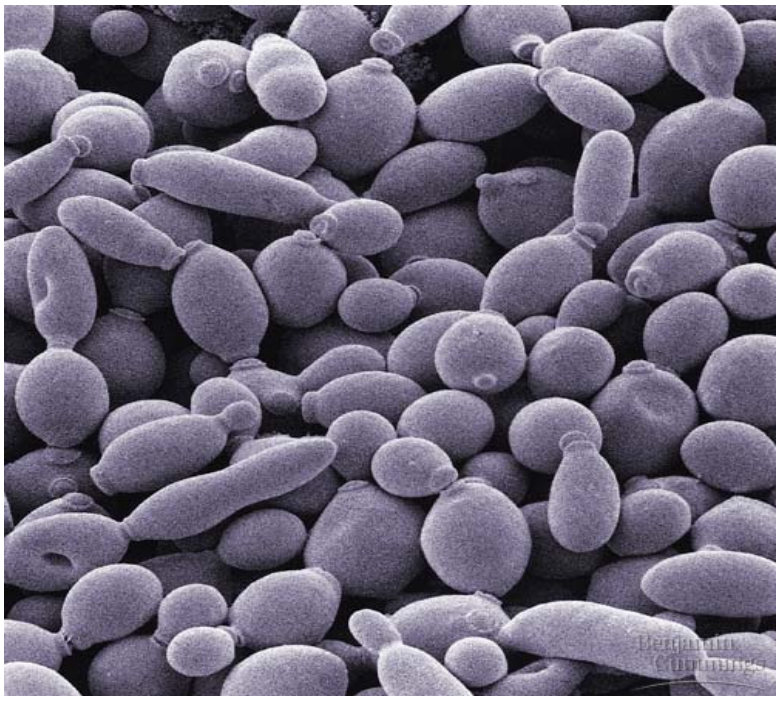

Figure 1-4 Yeast multiplication by budding. [17]

The second factor influencing the growth of gas bubbles is the gas retention capacity of the dough, which is a function of the strength of the gluten network. The other factors are the amount of carbon dioxide diffusing from the liquid phase into the nitrogen nuclei and the rate of bubble coalescence. [16, 18] The amount of energy used during mixing influences the bubble size distribution in the dough. Bubble size is important as it influences bubble growth during the proving stage and hence the texture of the final product. [18]

1.1.3 Baking

Baking is the last stage in bread making process; several chemical, biological and physical changes take place. For example oven spring (Tthe sudden increases of bread dough during the early stage of bread baking), protein denaturation, starch gelatinisation, formation of a porous structure, evaporation of water, crust formation, and death of yeast at temperature of 54ºC.[19, 20] These changes can take place in three different stages as shown in Figure 1-5. The first of these takes place during the first 6 minutes of baking, the temperature of the outer crumb increases to 60 ºC at rate of 4.7 ºC per minute, resulting in high enzymatic activity, high yeast growth rate, and expansion of the gases resulting in the oven spring. This effect takes place in the first 8 min of the baking time and reaches its maximum in the first 5 min resulting in increase in bread volume by one third. The

changes in matrix rheology with temperature have a significant effect on oven spring and bread quality. [21-24] Also in this stage, differences in baking quality and rheological properties between flours from different wheat varieties become more pronounced and are indicated by the extent of oven spring. [25] In the second stage, the combination of heat, moisture, and time induces starch gelatinisation which starts at approximately 65 ºC. [20] The heating rate and shear conditions during baking have a significant influence on starch gel characteristics, while there is limited shearing encountered during baking due to oven spring and gas expansion, heating rate becomes the most important factor in determining the characteristic of crumb structure. [26-30] In addition, heat setting of gluten proteins takes place in this stage, leading to the formation of the solid foam structure in the bread.

Changes in the transient glutenin protein network during baking are due to changes in protein surface hydrophobicity, sulfhydryl/disulfide interchanges, and the formation of new disulfide cross-links. [31-35] Also during this stage crust colour becomes more brown-gold in colour due to the chemical reactions producing coloured compounds, specifically the non enzymatic browning caramelisation and Maillard reactions. Caramelisation is a result of direct heating of carbohydrates, while the minimum requirements for the Maillard reaction are amino groups associated with proteins, reducing sugars, heat, and some water. [36-39] Browning depends on surface temperature, and is well correlated with weight loss during baking and oven temperature.[36, 40] This stage takes around 12 minutes and the temperature increases to 98 ºC at rate of 5.4 ºC per minute. [23] The last step involves volatilisation of organic compounds, which is considered to be the bake-out loss. This stage takes about 6 minutes.[23] During baking, heat transport towards the centre induces water transport and changes the water distribution in the bread loaf. [41]

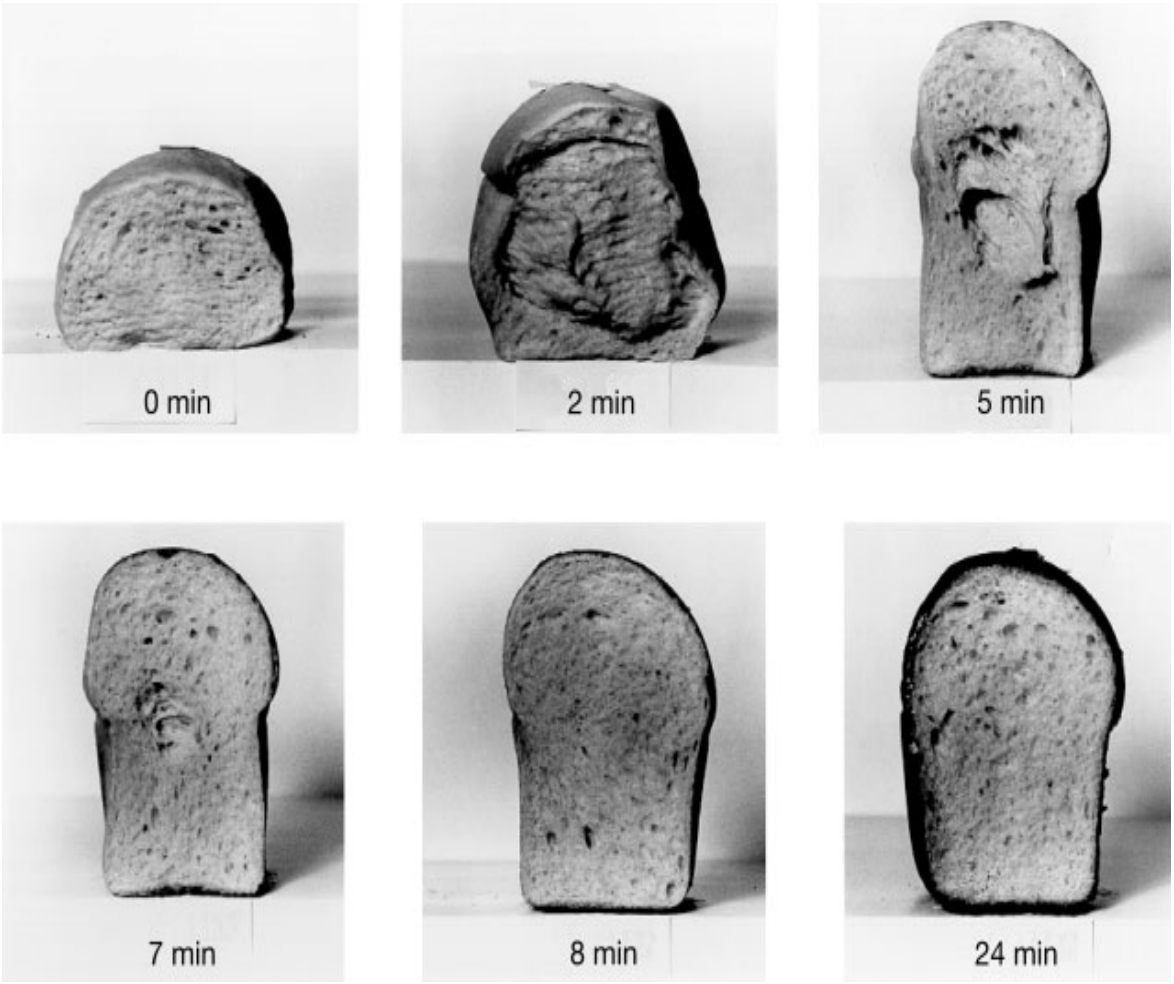

Figure 1-5 Different stages in the transformation of dough into bread during the baking process. [22]

1.2 Proteins

Proteins are polymers made up of amino acids, joined by strong covalent peptide bonds, where the amino group of one molecule reacts with the carboxyl group of the other molecule in a condensation reaction resulting in the elimination of water. [42-44] as shown in Figure 1-6.

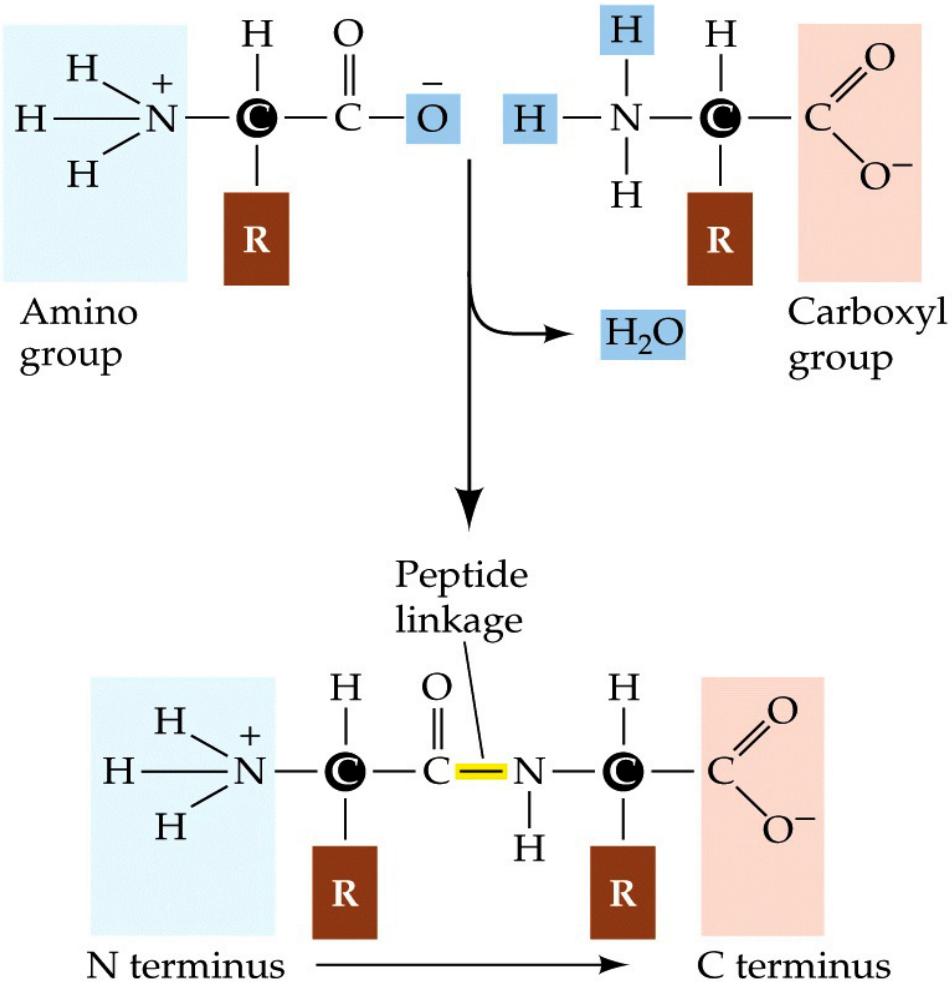

Figure 1-6 The formation of the peptide pond in proteins [43]

All proteins contain carbon, hydrogen, nitrogen, and oxygen atoms. Most also contain sulfur and some of have additional elements, including phosphorous in milk proteins and iron in myoglobin, as prosthetic groups. [45] Proteins are very important in foods, playing important roles in the food texture and adding nutritional value. [46]

1.2.1 Structure of amino acids

Amino acids are the building blocks of proteins. In all species proteins are made up of the same set of 20 standard amino acids. All of these are comprised of a central carbon atom (α-carbon) and attached to it are a carboxyl group (COOH), an amino group (NH_2), a hydrogen atom, and the side chain R which is specific to a particular amino acid [47, 48] as shown in Figure 1-7.

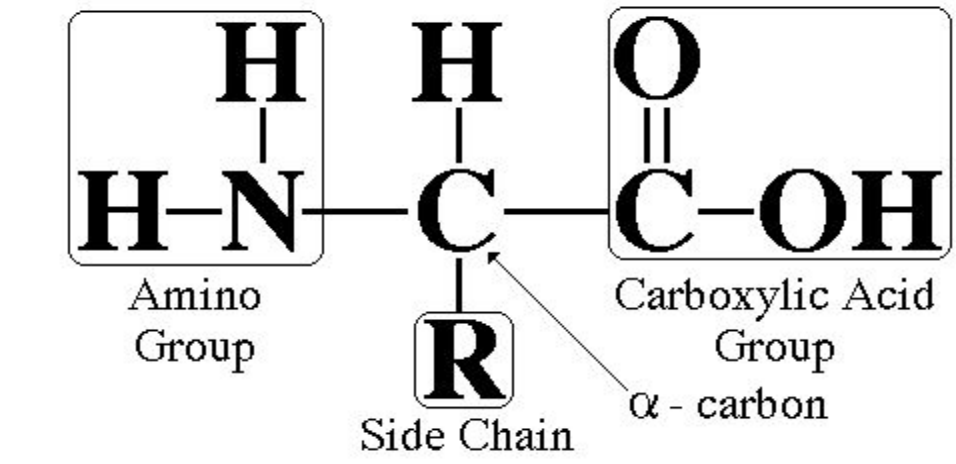

Figure 1-7 Amino acids structure [49]

The properties of all the 20 amino acids depend on the nature of their side chain. The simplest amino acid is Glycine in which the R group is a hydrogen atom. [44, 46, 48] The nature of the side chain and the structure of the amino acids are shown in Figure 1-8.

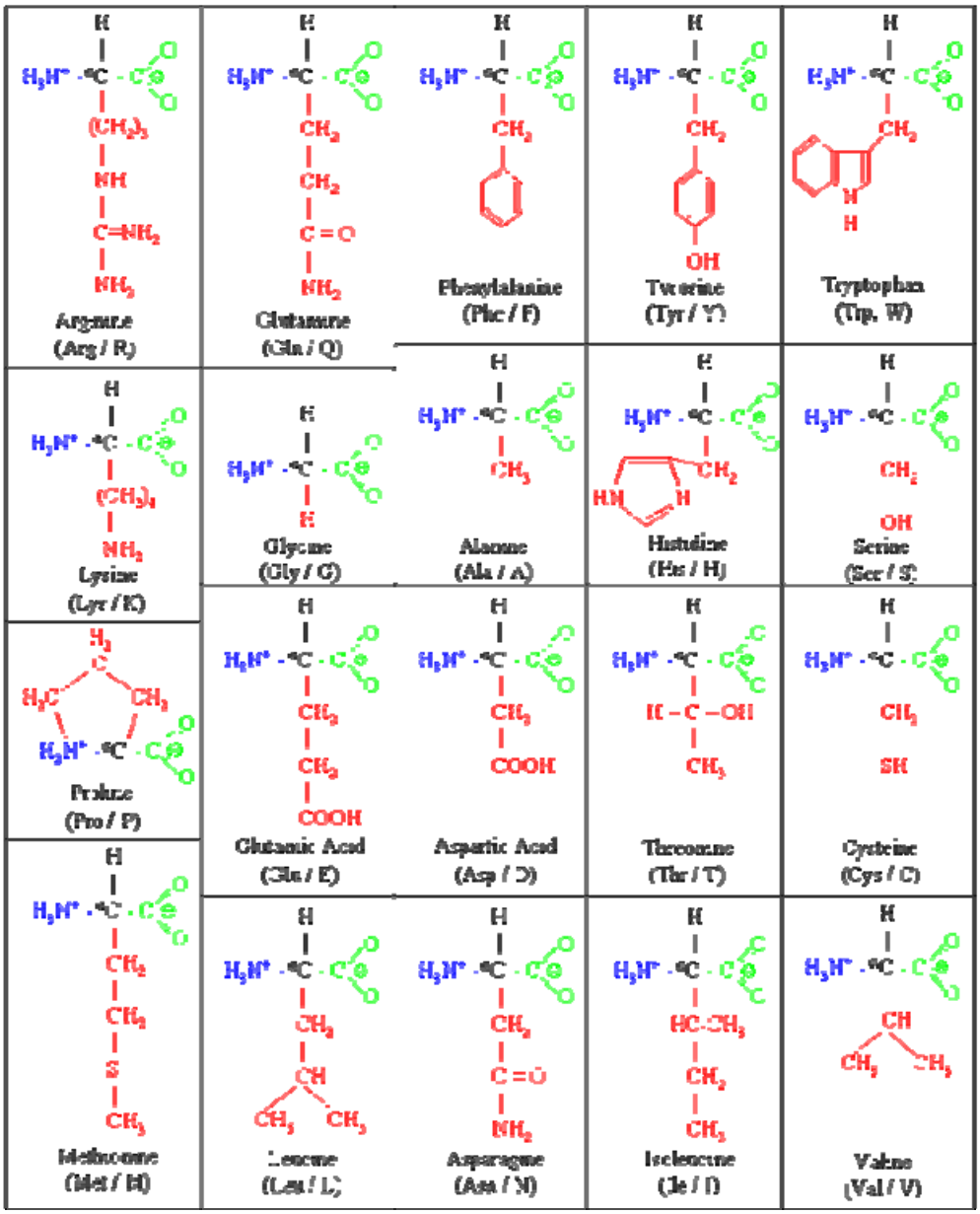

Figure 1-8 The structure of the 20 commonly occurring amino acids. [50]

The physicochemical properties of amino acids (polarity, acidity, basicity, aromaticity, bulkiness, conformational inflexibility, ability to form hydrogen bonds, ability to cross- link and chemical reactivity) are governed by the nature of their side chain. [47] Amino acids can be divided into four categories, according to the nature of their side chain. [46]

1.2.2 Hydrophobic or nonpolar amino acids

This group of amino acids contain nonpolar or hydrophobic (water “hating”) hydrocarbon side chains. Alanine is the simplest amino acid in this group, with a side chain consisting

of a methyl group (CH_3). Leucine and valine also belong to this group of amino acids, both contain longer, branched hydrocarbon groups. In proline the side chain is conformationally rigid. It is an important member of the nonpolar amino acid group, containing a bulky-five membered ring which interrupts ordered protein structure. Also belonging to this group is the sulfur containing amino acid methonine. This group of amino acids are involved in hydrophobic interactions in proteins whereby they associate with each other to avoid association with water. [42, 44, 46, 48]

1.2.3 Hydrophilic or polar amino acids

The amino acids in this group contain hydrophilic polar side chains. One example of amino acids in this group is cysteine, with its highly reactive hydrophilic sulfur containing side chain (SH). All amino acids in this group can form hydrogen bonds in proteins. Cysteine is unique because it can form the strong covalent disulfide bond when it interacts with another cysteine residue. As a result, the presence of cysteine in a protein has a significant effect on protein conformation. [42, 44, 46, 48, 51]

1.2.4 Negatively charged (acidic) amino acids

The side-chains of the acidic amino acids aspartic acid and glutamic acid are negatively charged at neutral pH because they both contain an additional carboxyl group. When an acidic amino acid is contained within a protein structure, the extra carboxyl group is free and may be charged depending on the pH of the medium. [42, 44, 46, 47, 52]

1.2.5 Positively charged (basic) amino acids

The amino acids arginine, lysine, and histidine are positively charged at pH 7 because they contain an extra additional group. This amino group is free, does not participate in peptide bond formation and may be positively charged depending on the medium pH.
Acidic amino acids and basic amino acids are involved in the formation of ionic interactions and salt bridges in proteins. [42, 44, 46, 47, 52]

1.3 Protein structure

A knowledge of protein structure is essential for understanding how the various proteins perform their function in food products. Experimental techniques for protein secondary

structure include X-ray crystallography, Nuclear magnetic resonance (NMR) spectroscopy and fourier transform infrared (FT-IR) spectroscopy. [53]

Proteins composed of single polypeptide chain, are generally considered at three levels of structure, the primary structure, the secondary structure and the tertiary structure, while proteins containing two or more polypeptide chains, each chain is a subunit and there is a quaternary level of structure. [42, 54] These levels of protein structure are presented in Figure 1-9.

1.3.1 Primary structure

The primary structure of polypeptide chain of a protein is the order in which amino acids are joined together in a chain and that will also determine the potential location of any disulfide bonds. [48]

1.3.2 Secondary structure

Within the folded protein a few characteristic patterns occur frequently due to the interactions between the chemical groups in the amino acids: these shapes are referred to as protein secondary structure. The patterns occur repeatedly because they are stable and the common protein secondary structures include α helix β sheet and β turns. [48, 55, 56]

1.3.3 Tertiary structure

Protein tertiary structure involves the folding and bending of the secondary structure. The final shape of a polypeptide is formed as a result of simple molecular forces set up by water- avoiding and water- attracting properties of the side chains. β sheet and α helix undergo twisting and folding until the hydrophobic groups are tucked to the inside of the polypeptide, away from water, and the hydrophilic groups remain exposed to the water. [42, 57]

1.3.4 Quaternary structure

Protein quaternary structure refers to the number and the arrangement of subunits that form a protein. Quaternary structure is an important protein attribute that is closely related to its function. [58]

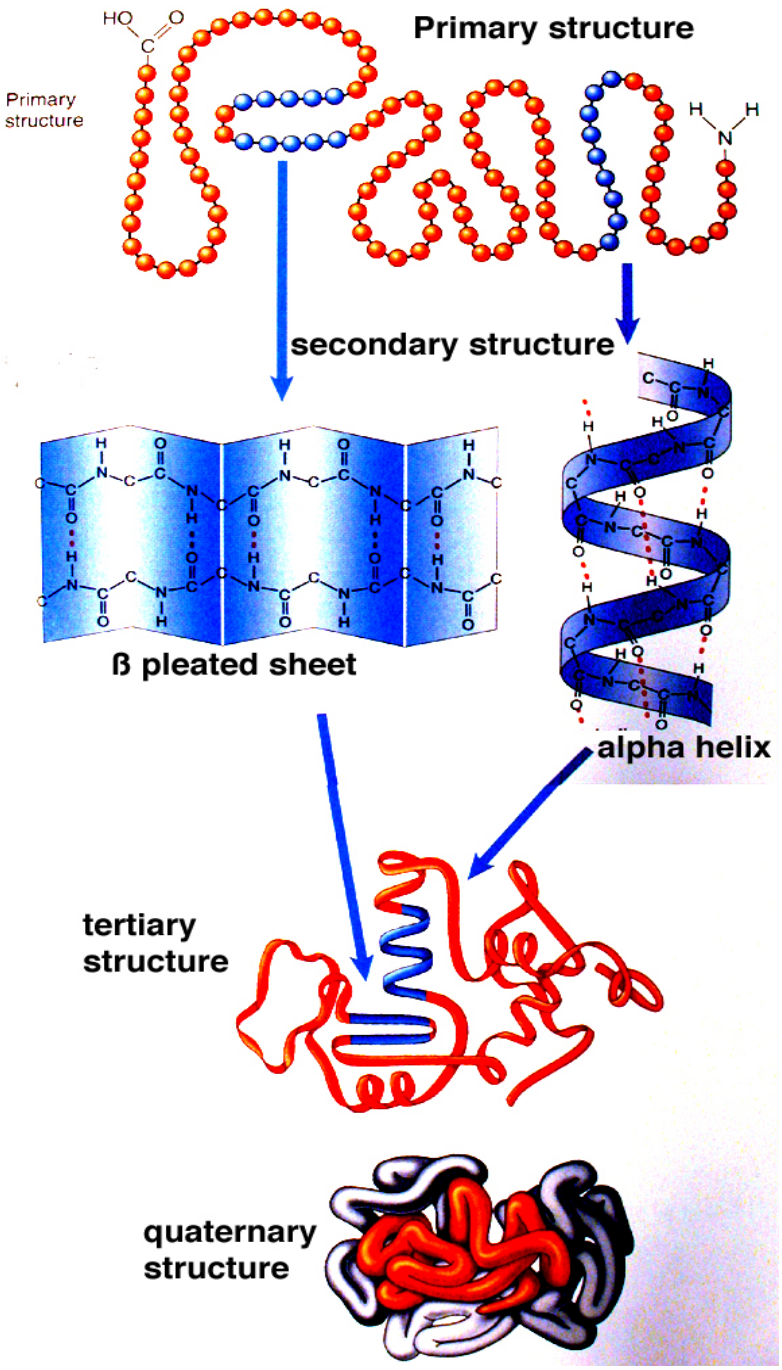

Figure 1-9 Protein structure. [59]

1.4 Forces stabilising protein structures

Protein primary structure is maintained by covalent bonds, while secondary, tertiary and quaternary structures are stabilized principally by noncovalent bonds. In addition, any disulfide bonds that form may be considered as contributing to the secondary and the tertiary levels of structure.[54] The –SH ends of two cysteine R groups often form an –S-

S- bond, a covalent disulfide bridge that typically brings distant parts of the chain together. [42, 51]

1.4.1 Hydrogen bonding

The properties of many substances suggest that, in addition to the normal chemical bonding between the atoms and ions, there exists some further interaction involving a hydrogen atom placed between two or more other groups of atoms. [60] Hydrogen bonding is a force of attraction between a hydrogen atom in one molecule and a small atom of high electro negativity in another molecule. It is an inter-molecular force rather than intera-molecular force. Hydrogen bonding plays an important role in the properties of water, protein and nucleic acids. [61] in the formation of the α-helix structure in proteins the hydrogen bonding causes the poly peptide to twist into a helical shape [62] as shown in Figure 1-10.

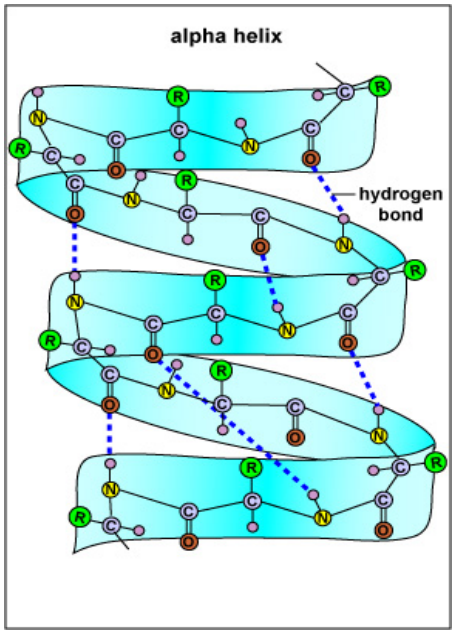

Figure 1-10 Hydrogen bonding in α helix. [16]

1.4.2 Hydrophobic interactions

These interactions arise because of the thermodynamic incompatibility between the nonpolar side chains of amino acids and water.[63, 64] The presence of the hydrophobic groups in an aqueous solution prevents the water molecules from forming hydrogen bonds and they interfere with the organisation of the water molecules. [52]

1.4.3 Electrostatic interactions

Among the various forces and interactions stabilising the tertiary and quaternary structures of proteins, electrostatic interaction is recognised as being as important as hydrophobic interactions in maintaining protein structure.[65] At neutral pH values, the side- chains of the acidic amino acids (aspartic and glutamic acids) will be negatively charged, while the side chain of the basic amino acids (lysine, arginine and histidine to some extent) will be positively charged. These groups may result in bonding between different portions of a given molecule or between two or more protein chains if appropriately positioned. [42, 52]

1.4.4 Van der Waal's forces

Van der Waal's forces are relatively weak forces that act over very short distances. They include both the attractive and repulsive components. The former involves interaction between induced dipoles formed by momentary fluctuation in the electron distribution in the nearby atoms. The later is when two atoms come so close that their electron orbitals overlap. The Van der Waals contact distance is that at which the attractive force is maximal and the repulsive force is minimal [48] as shown in Figure 1-11.

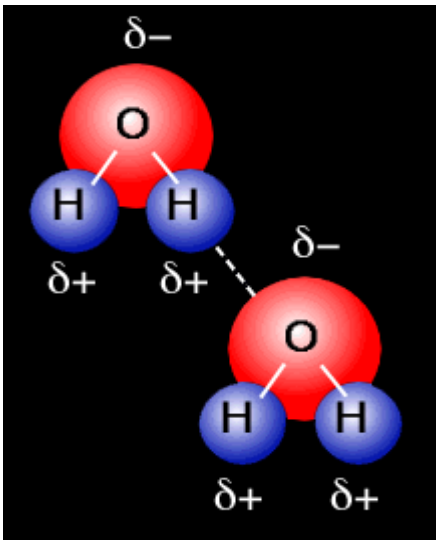

Figure 1-11 Van der Waal's forces. [49]

1.5 Infrared spectroscopy

The study of the interaction of infrared light with matter is referred to as infrared spectroscopy. The wave number range of mid infrared light in the electromagnetic spectrum is between 400cm^{-1}-4000cm^{-1}. [66] The nature of the radiation shown in Figure

1-13, has been interpreted by Maxwell's classical theory of electro- and magneto-dynamics. Radiation is considered as two mutually perpendicular electric and magnetic fields oscillating in single planes at right angles to each other and these are in phase and being propagated as sine waves [67] as shown in Figure 1-12.

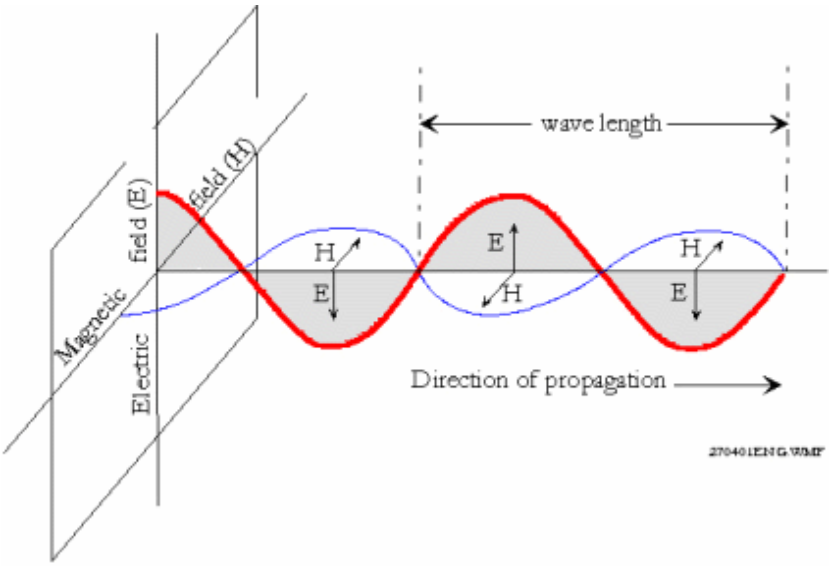

Figure 1-12 The electromagnetic wave. [68]

1.5.1 Interactions between molecules and light

The molecular energy of a specific molecule is the sum of its electronic energy, vibrational energy and rotational energy. Electronic energy is caused by visible light and ultraviolet energy, while vibration energy is caused by infrared radiation. Rotational energy is a function of near-infrared energy.[69, 70] There are five possible effects of the interaction between radiation and molecules, ultraviolet high energetic radiation can cause ionization, while electron transitions are usually caused by visible light. The other three possible effects are scattering, emission and absorption. The last of these is the process by which the energy of a photon is taken up by matter and this process plays a key role in (IR) spectroscopy [71] as shown in Figure 1-14.

The most unique physical property of a compound is its infrared absorption spectrum. No two compounds with different structures have the same IR spectra, with the exception of optical isomers. [72]

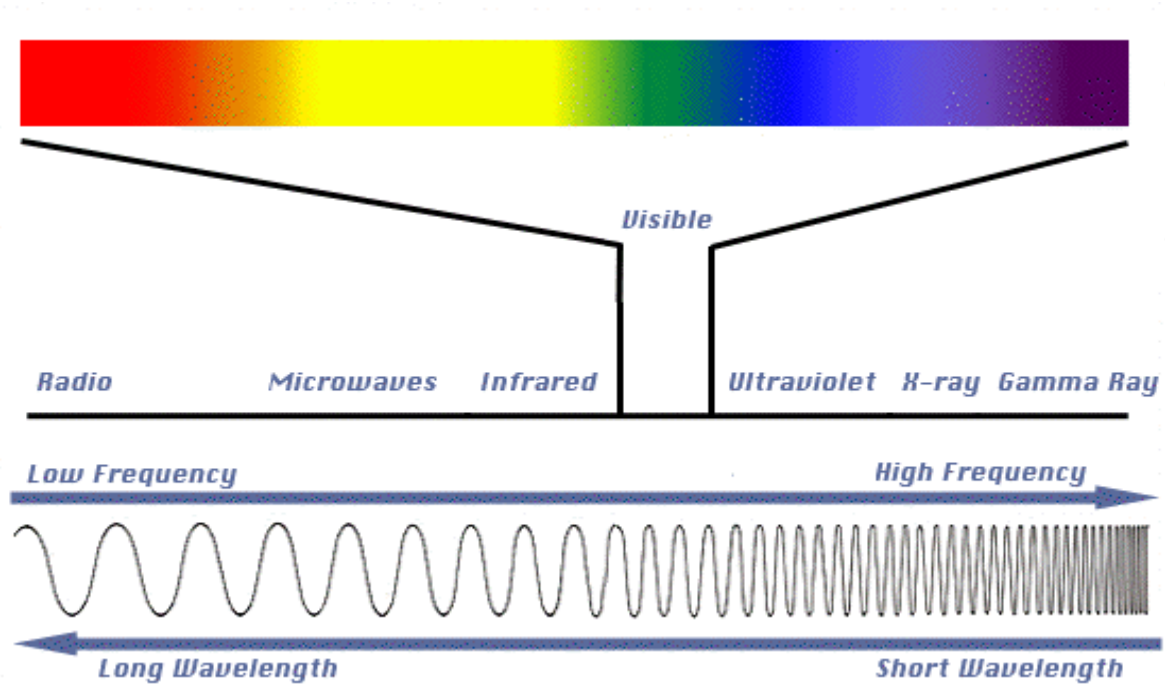

Figure 1-13 The electromagnetic spectrum. [73]

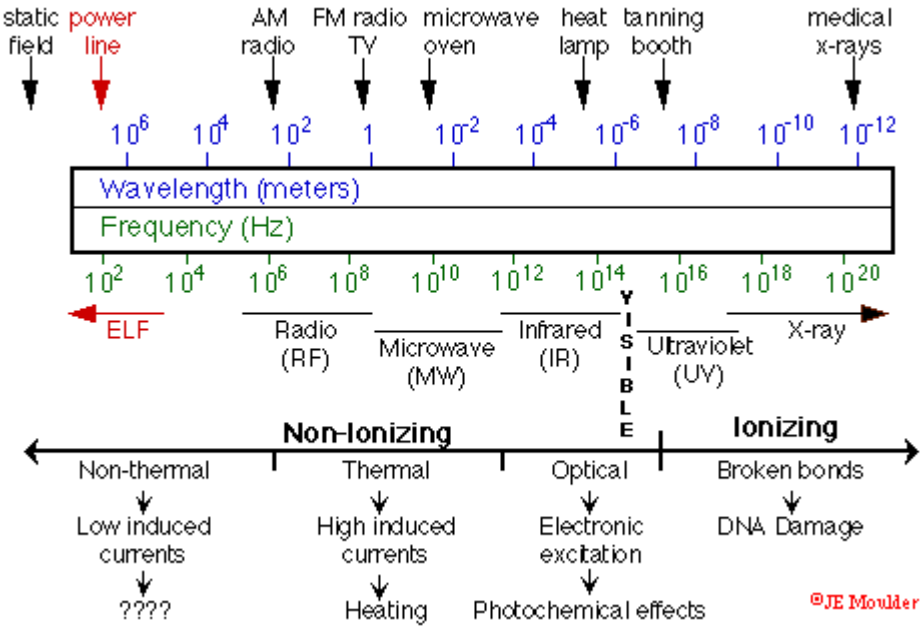

Figure 1-14 The effects of interaction between the electromagnetic spectrum radiation and molecules. [74]

1.5.2 FT-IR Measurements

The first step in producing an IR spectrum is to collect and store a reference interferogram with no sample in the sample cell. Then the sample is placed in the cell and a second interferogram is collected, the Fourier transformation is then applied to the two interferograms to compute the IR spectra of the reference and the sample. The ratio of the two spectra can then be computed to produce an IR spectrum of the sample. [67, 69, 72, 75, 76]

FT-IR measurement involves irradiating the sample with the whole range of IR frequencies; some of this irradiation will be absorbed by the sample if the radiant energy matches the energy of specific molecular vibrations and the remainder will be transmitted.[66, 71] The IR radiation in FT-IR measurement is guided by an optical device containing all the IR frequencies and this is referred to as an interferogram. In this the beam of light is divided into two different paths by a beam splitter, as described in Figure 1-15. The beam splitter is designed to transmit half of the radiation to strike the fixed mirrors, and reflect the other half to strike the moving mirrors [76]. An optical path difference is introduced between the two beams by translating the moving mirrors away from the beam spliter. The two beams are recombined at the beam splitter, after reflecting off their respective mirrors. After their recombination, half of the light is returned to the source and the other half is passed through the sample and then to a detector. [69, 71, 77-79] The resulting interferogram is then subjected to FT to produce the spectrum by changing the signal from the time domain to the frequency domain. [67, 69, 80, 81]

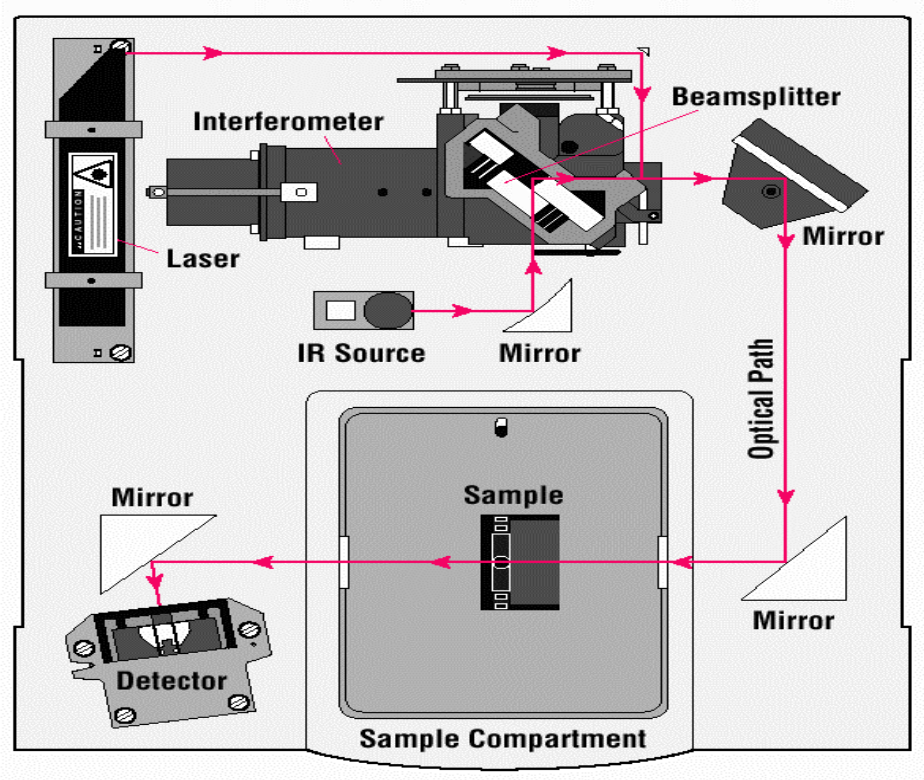

Figure 1-15 Schematic representation of an FT-IR spectrometer. [82]

1.5.3 Molecular vibration

The vibrational energy of a molecule is determined by the shape of the molecule, the masses of atoms and by the associated vibronic coupling.[71, 72] Each mass requires three coordinates to define its position, so for a molecule composed of N atoms there is 3n degree of freedom for its motion, six of which are translational and rotational of the molecule itself. This leaves 3n-6 or 3n-5 degrees of vibrational freedom if the molecular is linear.[66, 71, 82] The different types of vibration mode are shown in Figure 1-16 and are given descriptive names including asymmetric stretch, symmetric stretch, bending, rocking, twisting and scissoring. [48, 71, 72] The actual vibration of structure is a combination of all of the vibrational modes. [79, 81, 83]

For a vibrating molecule to absorb IR the molecular vibration has to cause a net change of the dipole moment of the molecule. Absorption strength depends on the size of deformation of the dipole moment due to vibration. [70, 77, 78, 84] A large deformation of the dipole moment results in strong absorption as in the case of C=O, while small deformation produces weak absorption as in the case of C-C. In addition, the strength of absorption increases with increasing polarity of the vibrating bond. [67, 72, 85, 86]

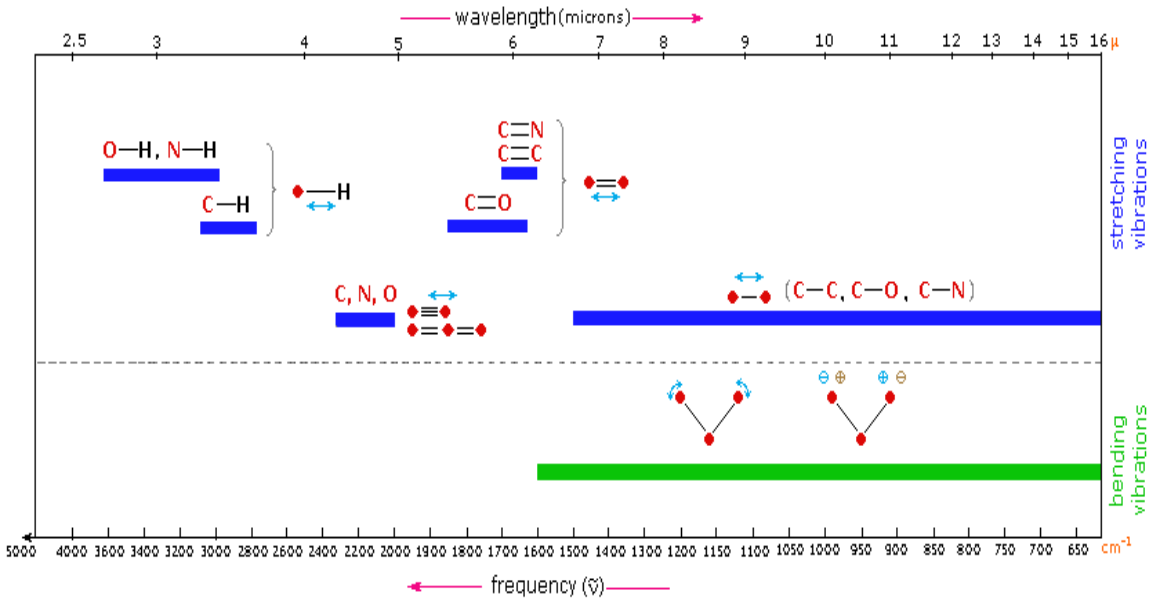

Figure 1-16 Stretching and bending vibrational bands in FT-IR spectrum adapted from. [87]

2

2 Literature review: I. Wheat flour components and baking ingredients

2.1 Wheat flour and its composition

2.1.1 Introduction

Cereal grains are important dietary sources of energy and a primary provider of proteins. [88, 89] Among the cereal grains, wheat is widely cultivated because of its relatively high protein, high carbohydrate, and mineral contents. [90, 91] Wheat is processed into a variety of different types of food products worldwide because of the unique viscoelastic properties of its dough.[92] The chemical composition of wheat greatly affects the end uses and the type of products which are made, as shown in Figure 2-1. Commercially three wheat species are grown *Triticum aestivum* (common wheat), *T. durum* (durum wheat) and *T. compactum* (club wheat). [91, 93]

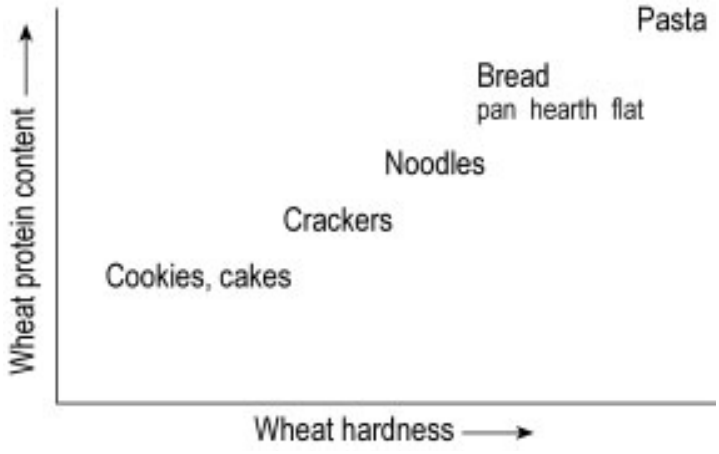

Figure 2-1 The relation between wheat hardness and protein content in relation to end uses [93]

Within Australia wheat is mainly used for human consumption, with bakers using approximately half of the domestic flour production. The other main group of users is the starch/gluten industry which uses about a quarter of the domestic flour production. [94]

2.1.2 Composition of wheat

Several factors govern the composition of wheat grains and result in variations from one area to another and from year to year. [95] Table 2-1 shows the ranges of major components in wheats grown in the U.S. The different layers of wheat kernel are shown in Figure 2-2 . The seed coat encloses the endosperm and the germ in the wheat kernel. The aleurone layer is rich in soluble protein and contains fat, enzymes, and minerals. Flour is essentially a product derived from the endosperm; its thin –walled cells are packed with starch granules embedded in a protein matrix. [91]

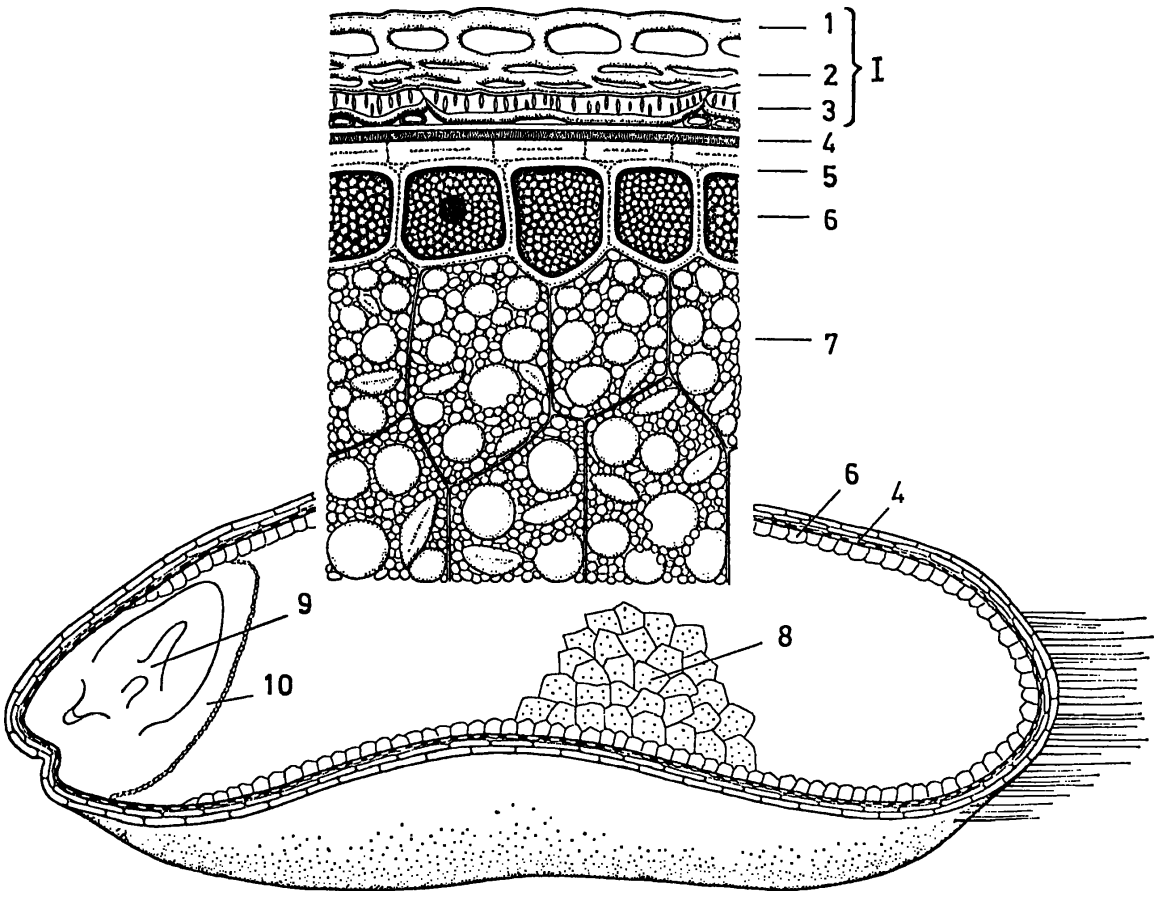

Figure 2-2 Longitudinal section of a wheat kernel showing the different layers; 1. Pericarp, 2. Hypodermis, 3. Tube cells, 4. Seed coat, 5. Nucellar tissue, 6. Aleurone layer, 7. Outer starchy endosperm cells, 8. Inner starchy endosperm cells, 9. Germ, 10. Scutellum, from [91]

Table 2-1 Ranges for major components in U.S. wheat [95]

Grain component	Range %
Protein (Nx5.7)	7.0-18.0
Mineral matter (ash)	1.5-2.0
Lipids (fat)	1.5-2.0
Starch	60.0-68.0
Cellulose (crude fibre)	2.0-2.5
Moisture	8.0-18.0

2.1.2.1 Starch

Starch is the energy reserve carbohydrate of many plants. [96, 97] Like most foods, wheat contains starch as a macro-constituent, typically supplying 50-70% of the energy in the human diet. [98]

Amylose and amylopectin are the two distinct constituents of starch; glucose is the monomer building block in both of the forms of starch molecules [99, 100] as shown in Figure 2-3.

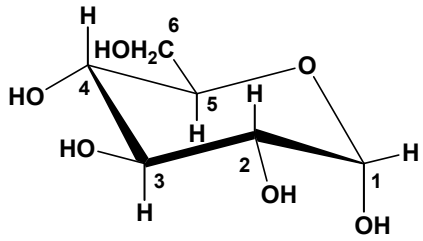

Figure 2-3 α-(D) glucose

Amylose is a linear polymer, consisting of α-(1,4)-linked D-glucopyranosyl units, its degree of polymerisation ranging from 500-6000 glucose units. [101] Figure 2-4 illustrates the linear structure of amylose.

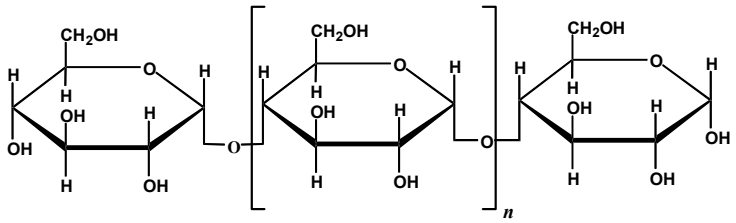

Figure 2-4 Amylose structure where n indicates a relatively large number

Amylopectin is a branched glucose polymer typically with a molecular weight around $10^{7,}$ it contains chains of 20-25 glucose units connected via α-(1,4) glucosidic linkage in the linear regions and interlinked by α-(1,6) glucosidic linkage in the branched regions.[97] The structure of amylopectin is presented in Figure 2-5.

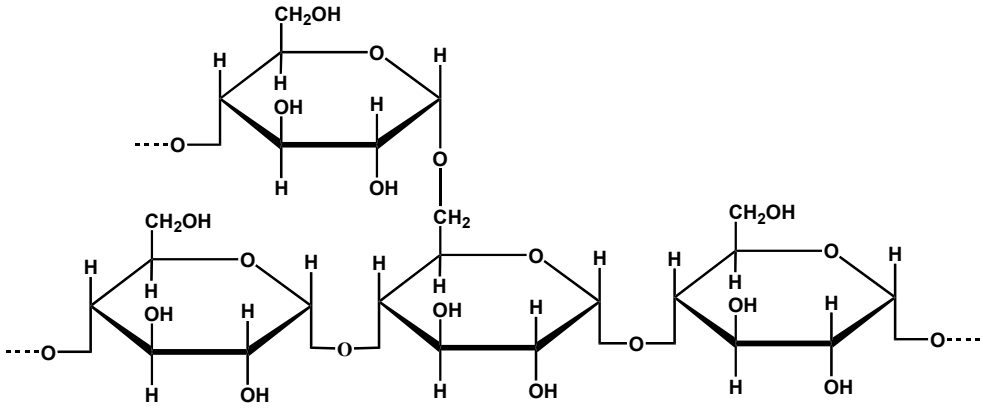

Figure 2-5 Amylopectin structure showing the α-(1,6) linkage at a branch point in the polymeric structure

2.1.2.1.1 The ratio of amylose and amylopectin

The end uses of many cereal starches and their gelatinisation, solubility, formation of resistant starch, gelation characteristics and physicochemical properties are influenced by the ratio of amylose to amylopectin. [102] In addition, the molecular weight of both amylose and amylopectin and the molecular fine structure affect the physicochemical characteristics of starch from a particular source. [103] Different starches contain different amount of amylose and amylopectin, but generally the typical levels are 25-28% amylose and 72-75% amylopectin. [101] The waxy varieties of some cereals including rice, maize and sorghum contain as much as 99% amylopectin. [97]

2.1.2.1.2 Starch gelatinisation

Starch undergoes irreversible changes when heated in the presence of water and the term gelatinisation is used to refer to these changes. [104] Gelatinisation is initiated with the swelling of the starch granular followed by an increase in the molecular mobility to an extent sufficient to disrupt the crystal structure within the starch granule, ultimately resulting in the formation of amorphous paste or gel upon cooling. [105]

2.1.2.1.3 Starch crystal and amorphous structure

The structure of starch molecules within intact granules is described as semicrystalline and crystallisation occurs in two polymorphic forms, known as the A and B-type structures. [106]

Wheat starch, like that from most cereal grains contains crystal structure type A which consists of starch double helices packed into a monoclinic array. [107] The B-type pattern is believed to involve double helices parallel-packed into hexagonal unit cells, and this is found in tuber starches. [106] Amylopectin is structurally highly organized, with regions having a high- frequency of branching alternating with others that are devoid of branches thereby enabling intervening linear chains to align in parallel arrays of double helices, [96] as shown in Figure 2-6.

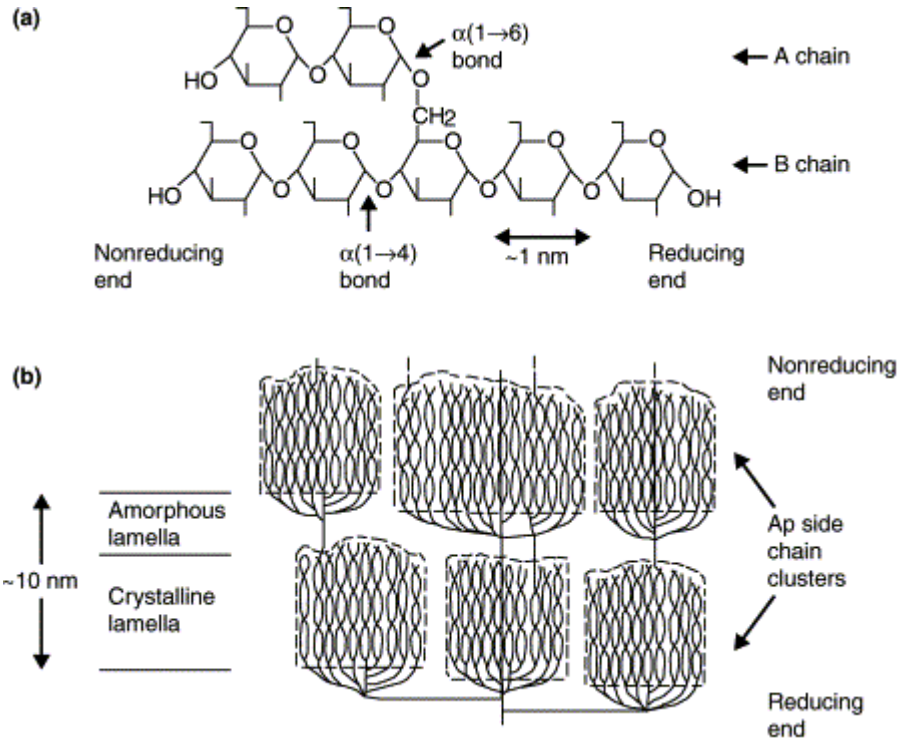

Figure 2-6 Amylopectin structure (a) glucosyl units connected via α-1,4 and α-1,6 glycoside linkages (b) the cluster model of amylopectin structure [96]

2.1.2.1.4 Starch damage

During the milling process whereby wheat is processed into flour, the action of the mill rollers causes physical damage to a relatively small proportion of the starch granules of the flour. This is significant in the subsequent utilisation of the flour because when the crystallites are disrupted, the starch granules can swell in water at room temperature. [93]

The milling of hard grained wheat varieties produces a higher level of starch damage than is found for softer varieties of wheat.

The levels of starch damage in wheat flour can be assessed by two different approaches. The first is the measurement of the susceptibility of a sample of the flour to amylolysis, the second is the extractability of starch measured as the amylose content of a cold aqueous extract of the flour. [108]

2.1.2.2 Protein

Wheat flour protein is unique among the proteins of both cereal grains, as well as other food ingredients, in its ability to form dough with viscoelastic properties, suitable for processing of many appealing food products including breads, biscuit and pasta products. [90]

Gluten is the term used to describe the matrix which forms during dough development and this largely consists of the wheat seed storage prolamins. The first scientific reports on the fractionation of wheat protein were provided by Osborne, who carried out experiments by extracting flour with salt solution. By this approach gluten can be separated from starch. [109] The prolamin components are the major storage proteins in wheat endosperm. [110] In addition to the proteins which account for 80-90%, gluten also contains 3.5-6.8% lipids, 0.5-0.9 minerals, and 7.0- 16% carbohydrates. [111]

The wheat prolamins consist of the glutenins which are polymeric and the monomeric gliadins. [112] The gliadin molecules are soluble in aqueous alcohols while glutenin is not soluble, because it is a disulfide-stabilised polymer. The reduction of the disulfide bonds in glutenin molecular allow the separation of high molecular weight glutenin (HMW) and low molecular weight glutenin (LMW) components. [113] The disulfide cross-links play a highly significant role in the physico-chemical and functional properties of wheat gluten. [92] Figure 2-7 demonstrates the role of the disulfide linkage in gluten components. [114] Figure 2-8 shows the formation of the disulfide bond during dough mixing. [29, 91]

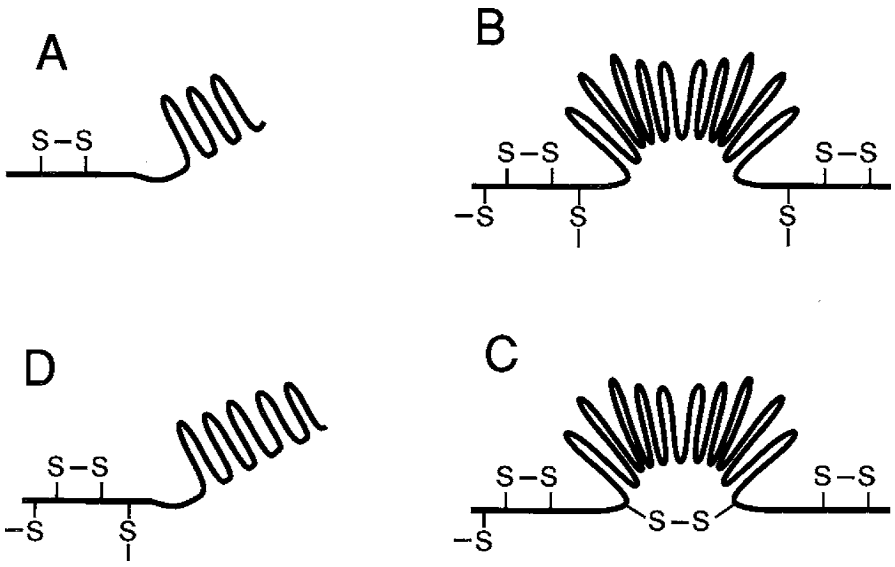

Figure 2-7 Schematic depiction of gluten components [114]. Note the clockwise presentation of the diagram (A) gliadin; (B) HMW glutenin sub unit showing action of β spirals as molecular spring; (C) HMW glutenin sub unit showing the action of the disulfide in preventing the extension of β spirals; (D) LMW glutenin sub unit.

The monomeric gliadins are classified into four groups based on the molecular mobility during gel electrophoresis under conditions of low pH and these are commonly referred to as α-, β-, γ- and ω- gliadins in order of decreasing mobility. [115] In this context, the baking potential of wheat flour depends largely on the protein quality and quantity. [116] Gliadin confers viscous properties on gluten, while glutenin is responsible for the strength and elasticity of the gluten network. This is necessary for the gas retention capacity of the dough. [109, 111, 117, 118] The composition of glutenin fractions and the ratio of glutenin to gliadin are the primary determinants of the viscoelastic properties of wheat gluten and therefore, also of the bread making quality of a flour. [111, 113, 117]

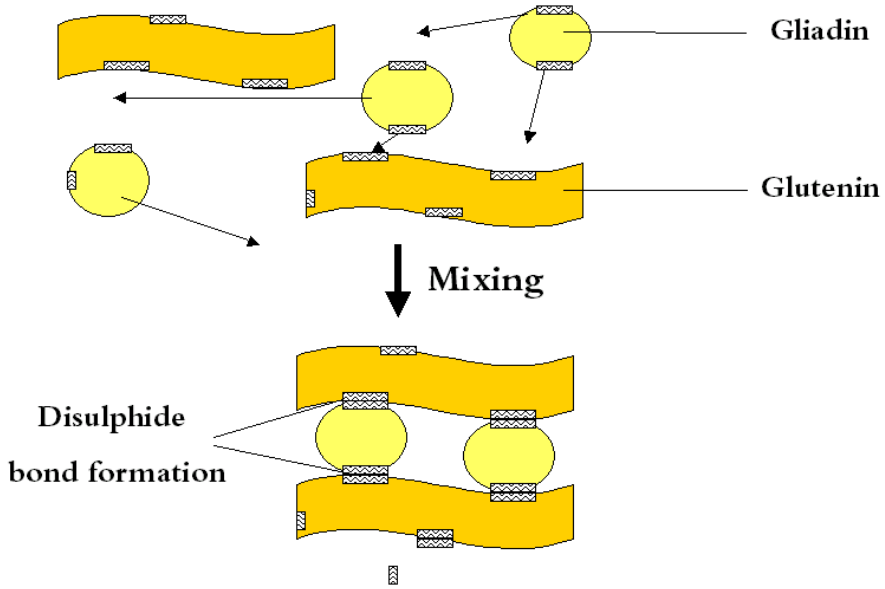

Figure 2-8 The effects of formation of disulfide bonds between glutenin and gliadin during the dough mixing [29]

2.1.2.3 Lipids

Lipids represent a relatively small proportion of wheat flour accounting for approximately 2.5%, with many different components making up this group of relatively hydrophobic molecules. The non polar lipids (triacylglycerols, diacylglycerols, free fatty acids and sterol esters) comprise about 1% and the main polar lipids being phospholipids (0.9%) and galactosyl glycerides (0.6%). [114] During the baking process lipids interact with other flour components especially proteins and these interactions have a significant influence on the bread making quality of flour. [119] Polar lipids, the acylglycerols are known to have an impact on enhancing loaf volume, while phospholipids can only contribute to improved bread volume in the presence of added shortening. [119, 120]

2.1.2.4 Non-starch polysaccharides

Non-starch polysaccharides are plant polymers which cannot be hydrolysed by mammalian α-amylases. They typically contain combinations of various hexoses and pentoses particularly including arabinose, xylose, mannose, glucose and galactose as the principal constituent sugars. [121] Non-starch polysaccharides in wheat flour are considered to be minor components accounting, in total for only 2.0-2.5% of flour. [114] Arabinoxylan (AX) is the principal non-starch polysaccharide in wheat flour and it is known to have very high water binding capacity. [122, 123] Depending on their water extractability, non-starch polysaccharides can be divided into water extractable

pentosans, (WEP) which generally account for 25% of all pentosan and those that are not extractable using water (the remaining 75%). [123, 124] Water soluble pentosans, (W-SP) are believed to have an impact on bread volume, crumb texture and bread staling. [123, 125, 126] Figure 2-9 shows the effect of different AX fractions on bread volume. [101]

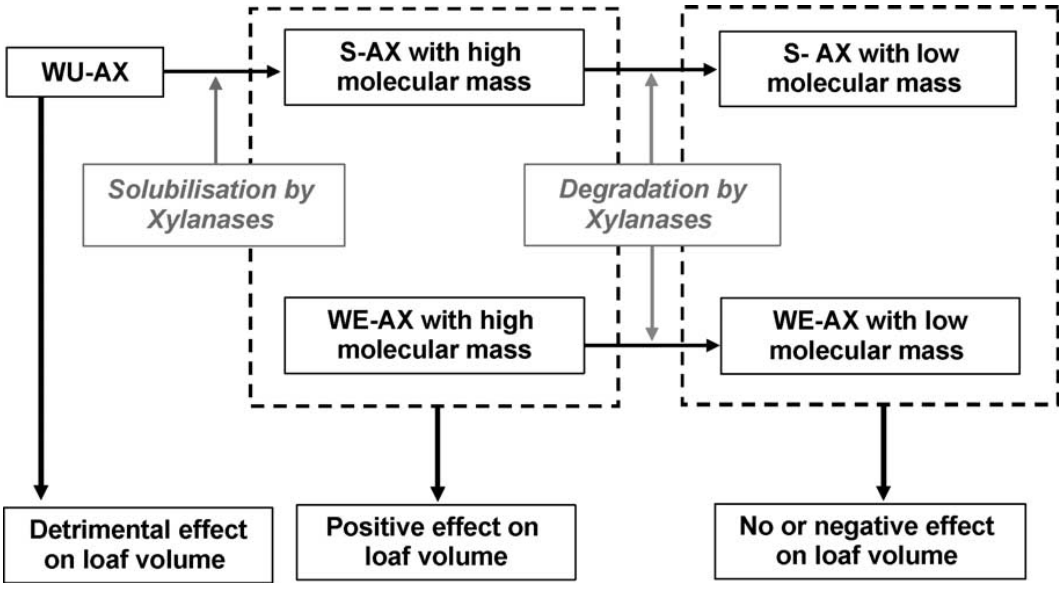

Figure 2-9 The effect of different AX fractions on bread volume [101] Note that the abbreviations used here are: 1. WU-AX water unextractable arabinoxylan 2. S-AX soluble arabinoxylan 3. WE-AX water extractable arabinoxylan.

AX-bound ferulic acid is a component of WEP, and it is reported to be involved in the direct effect of WEP on gluten formation and in the lower extensibility of dough and gluten. [123, 127] The free radical generating oxidant causes AX to undergo oxidative gelation through the dimerisation of esterified ferulic acid [114, 126] as shown in Figure 2-10.

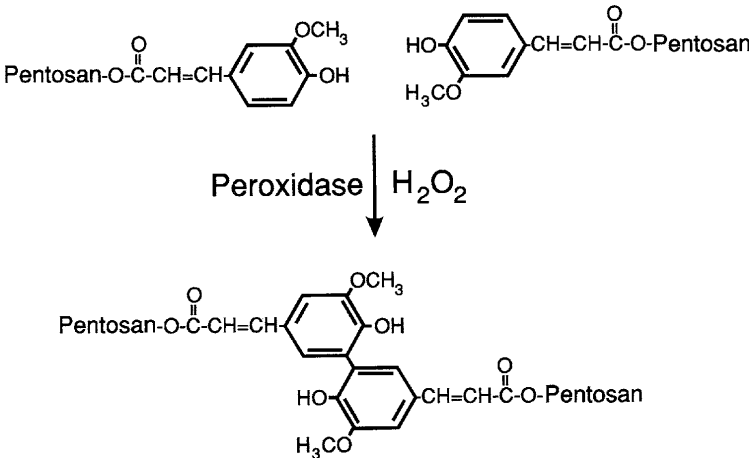

Figure 2-10 The oxidative cross linking of ferulic acid in pentosan [114]

2.1.2.5 Ash

Generally the overall ash content of wheat grains is in the range of 1.5-2.0%. Ash components in the wheat kernel are distributed unevenly; the bran contains 6% while in the level in the endosperm is much lower, typically 0.4%. [128] These ash contents can vary depending on grain hardness, environmental conditions and the variety of the wheat. [129] The presence of elevated levels of ash in wheat flour indicates contamination of the flour with bran particles, which have detrimental effect on the baking potential of a flour.[93]

2.2 Baking ingredients

Bread is considered to be a very important staple food in western countries. [130] Depending on the type of the bread, the process used, flour type and end product specification, different additives and ingredients can be incorporated into the bread formula, in order to enhance the eating quality and appeal of the product or to improve the keeping quality of bread. [130, 131] The chemical composition of the flour and the quality and the quantity of ingredients used are of great importance for the characteristics of the baked bread. [132] There are three main stages in bread-baking processes and these are dough mixing, fermentation and baking. [133]

2.2.1 Yeast

Saccharomyces cerevisiae or bakers yeast is widely used in the production of different types of bread as well as other food products. The main function of incorporating yeast

into a bread formula is to generate gas during the fermentation process as a part of the metabolic activity of yeast. [95] For this, yeast utilizes sugars which may be either added directly to the dough formula or be produced by the action of amylases during fermentation, to produce ethanol and carbon dioxide according to the equation below. [93, 95]

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$$

The types of yeast used in bread making can be classified into two classes based on their stability and the way they are processed. These are the fresh forms of yeast, which can be compressed, crumbled or cream types, and the second class are the dry forms and these may be instant or active dry yeast. [95, 134]

The selective permeability property of the yeast cell wall plays an important role in regulating the transportation of the required nutrients from the dough medium into the cell and these include sugars, amino acids, as well as other nutrients. Additionally, the cell wall contributes through the release of the metabolic by-products including carbon dioxide and alcohol from the cell into the dough medium. [135] The different parts of the yeast cell are illustrated in Figure 2-11.

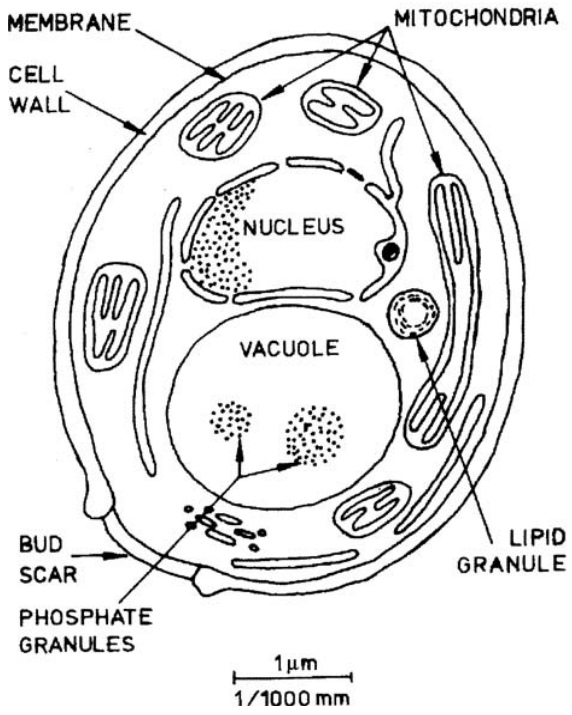

Figure 2-11 The yeast cell. [135]

2.2.2 Salt

In addition to its contribution to the taste of bread, salt serves so many other functions in the bread making process. It tightens the gluten network structure resulting in stronger dough and this is then better able to retain the gas produced by the yeast. Also its strength effect on the dough could be due to the inhibitory action on proteases.[54] Some salts favour the hydrolytic action of amylases on starch, thereby contributing to the maintenance of a continuous supply of simple sugars in the form of maltose for yeast growth. [136] Salt probably also regulates yeast activity during the fermentation by competing with yeast for water, resulting in moderate expansion of the dough without rupturing the gluten network. [54, 136]

2.2.3 Shortening

The term shortening is usually used in the baking industry to describe fats, oils, their derivatives as well as blends of these [136, 137] The functional significance of native wheat flour lipids and of added lipids as shortening during bread making has been comprehensively investigated. [138] They are used in the baking process to enhance bread volume and crumb softness, as illustrated in Figure 2-12. [136, 137] Shortening lubricates the gluten strands, preventing these from sticking to each other and by doing so the gluten strands are shortened. [57]

Figure 2-12 Effect of different levels of fat incorporation on bread volume [135]

2.2.4 Bread improver

The term improver is widely used in the baking industry, and it refers to a group of ingredients that in some way contribute by enhancing the baking performance, sensory attributes and storage stability of bread. [139] Bread improvers include enzymes, emulsifiers, oxidizing agents, reducing agents, nutrients for yeast growth as well as other ingredients. However, a minimally processed high quality food with few or no additives is the preference of most modern consumers. [139, 140]

2.2.4.1 Enzymes

Enzymes are proteins possessing a powerful catalytic activity. They are synthesized by biological cells to catalyse chemical reactions related to the metabolic requirements of the cell and the organism. [141] Their properties are important to food chemists since they are available in increasing number both for use as specific tools and reagents in food analysis [142] and also for incorporation into food formulations and use in industrial food processing. [143-147]

2.2.4.1.1 Enzyme specificity

One of the significant attributes of enzymes is that most are highly specific for a particular reaction. Most enzymes specifically bind to only one or a very limited number of the substrates during activated so that, among the several thermodynamically permissible reactions, only one can specifically occurs. [143] The catalytic activity of an individual enzyme is due to its particular protein structure. A specific chemical reaction is catalysed at an area representing a relatively small portion of the surface of an enzyme and this is called the active site. There is chemical and physical interaction occurring at this site to catalyse a particular chemical reaction that certain enzyme. [141, 145, 148, 149]

2.2.4.1.2 Nomenclature of enzymes

The nomenclature committee of the International Union of Biochemistry and Molecular Biology (IUBMB) has developed a system in which all enzymes are classified into six major groups, based on the nature of the chemical reaction catalysed. [141, 143, 145, 149]

1. Oxidoreductases

1.1 Acting on =CH-OH group of substrates

1.1.1Requires NAD+ or NADP+ as hydrogen acceptor

1.1.1.1 Specific substrate is ethyl alcohol

2. Transferases

2.1 Transfer of methyl groups

2.2 Transfer of glycosyl groups

3. Hydrolases
4. Lyases (cleave C-C, C-O, C-N, and other groups by elimination, leaving double bonds, or conversely adding groups to double bonds).
5. Isomerases (involved in the catalysis of isomerisation reactions within one molecule).
6. Ligases (involved in the biosynthesis of a compound with the simultaneous hydrolysis of the pyrophosphate bond in ATP or a similar triphosphate. [141, 143, 145-148]

2.2.4.1.3 Enzymes in baking

Enzymes have a very central role in the baking industry, with many composite bread improver formulations containing a number of ingredients which are sources of enzymes. Among the enzymes used most widely in the baking process are amylases, proteases, lipoxygenase and hemicellulases [140, 144] as shown in Table 2-2.

2.2.4.1.4 Amylases

Amylases are routinely incorporated into the formulations of breads of many styles including both loaf products as well as many of the flat breads popular in the Middle East. Their functionality depends primarily on their specificity as shown in Figure 2-13, as well as the degradation products and their thermostability. [101] α-Amylases are categorised as endoglucanases on the basis that they hydrolyse internal α-1,4 or α-1,6 linkages by random attack. On the other hand, β-amylases and glucoamylases are exoglucanases because they act by attacking only those linkages occurring at the end of a chain of monosaccharide units, specifically from the non reducing end. [144, 150, 151]

The functions of α-amylases include their action as anti firming agents because it is has been shown that some bacterial α-amylases decrease the rate of bread firming. [152] Also it has been reported that barley malt α-amylase increases the rate of bread firming while fungal α-amylase decreases the initial firmness of bread. [153] Different mechanisms for the role of different α-amylases as antifirming agents were proposed; these reflect either

the effect of released dextrins or the modified residual starch, impacting the rheological properties of the dough and subsequently starch retrogradation. [144, 154]

Table 2-2 The main enzymes used in cereal grain processing, their reactions, and their substrates [144]

Class of enzyme	Substrate(s)	Reaction catalysed
Amylolytic enzymes	Starch	Hydrolysis
α-Amylase EC 3.2.1.1	Amylose and amylopectin	α(1→4) D-glucosidic linkage endo mode of action
β-Amylase EC 3.2.1.2	Amylase and amylopectin	α(1→4) D-glucosidic linkage exo mode of action
Glucoamylase EC 3.2.1.3	Amylase and amylopectin	α(1→4) and α(1→6) D-glucosidic linkage
Pullulanase EC 3.2.1.41	Amylopectin	α(1→6) D-glucosidic linkage
Cellulases & hemicellulases	Cellulose, β-glucan & pentosans	Hydrolysis
Cellulase EC 3.2.1.4	Cellulose & β-glucan	β(1→4) glucosidic linkage
Laminarinase EC 3.2.1.6	β-glucan	β (1→4) and β (1→3) D-glucosidic linkage
Lichenase EC 3.2.1.73	β-glucan	β(1→4) D-glucosidic linkage
Endo 1,4- β-D-Xylanase EC 3.2.1.8	Arabinoxylan	β(1→4) D-Xylosidic linkage
Ferulic acid esterase EC 3.1.1.1	Arabinoxylan containing ferulic acid groups	Ester bonds to liberate ferulic acid
Proteases	Protein	Hydrolysis of peptide bonds
Lipases and esterases	Lipids and phospholipids	Hydrolysis of ester bonds
Lipase EC 3.1.1.3	Triacylglycerol	Liberation of free fatty acids
Lysophospholipase EC 3.1.1.5	lysophospholipids	Liberation of carboxylic acids
6-phytase EC 3.1.3.26 3-phytase EC 3.1.3.8	Phytate	Liberation of phosphate group
Oxidases	Various substrates	Oxidoreduction with oxygen as electron acceptor
Lipoxygenase EC 1.13.11.12	Poly unsaturated fatty acids	Oxidation of fatty acids
Glucose oxidase EC 1.1.3.4	Glucose	Oxidation of glucose to produce hydrogen peroxide

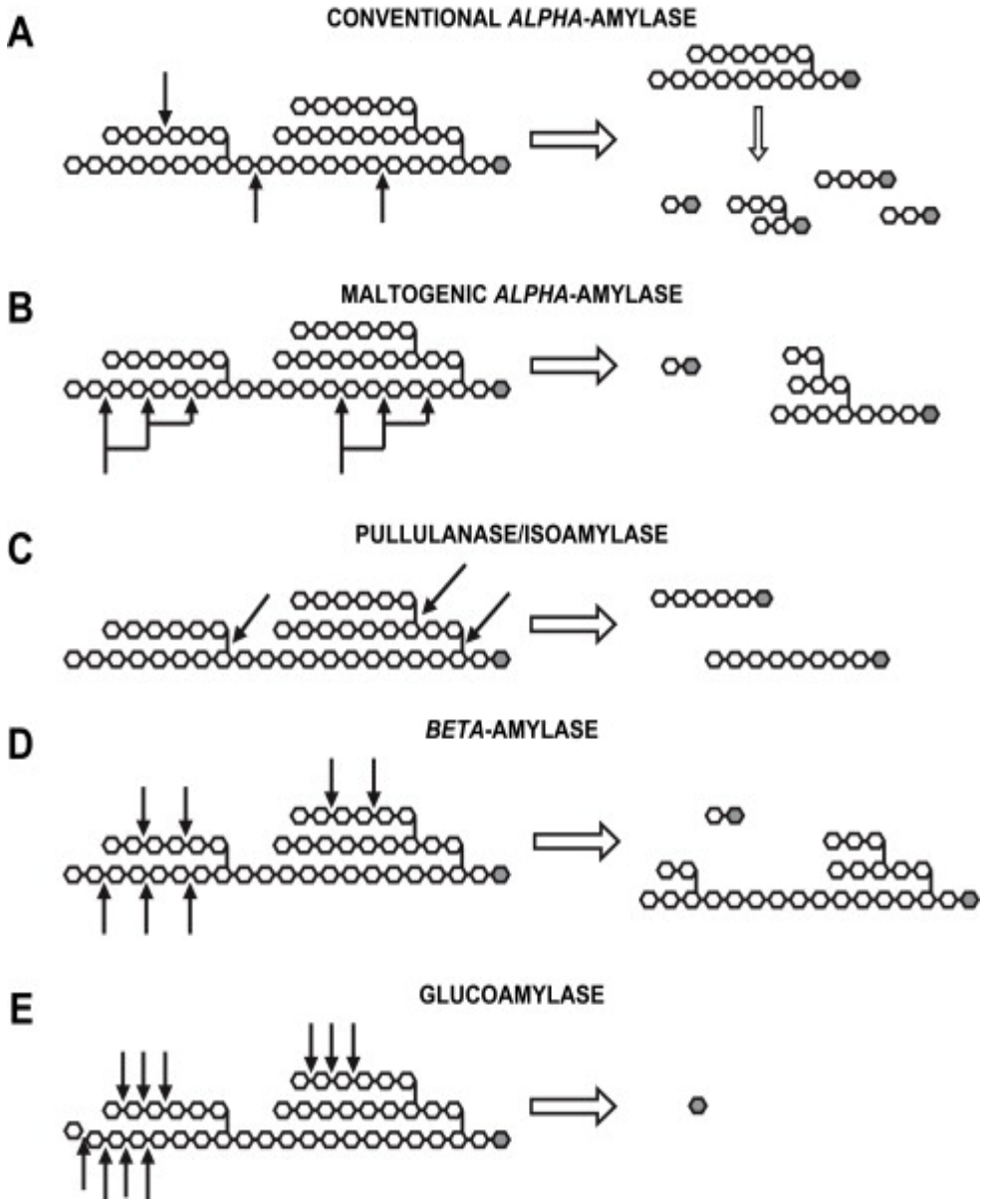

Figure 2-13 The action of different types of amylolytic enzymes on amylopectin polymer [31]

2.2.4.1.5 Proteases

Wheat proteases belong to the papain type of enzymes which are activated in the presence of compounds that reduce the disulfide bonds. While native proteases have little effect on bread quality, gluten degrading proteolytic enzymes have been found to have a drastic effect on bread quality. [140]

2.2.4.1.6 Hemicellulases

Hemicelluloses are widely distributed heteropolysaccharides, and there is a diverse group of enzymes that are involved in their degradation. One example is the xylanases and these are increasingly used in the baking industry to enhance dough quality and facilitate the increase in dough volume required to achieve the optimal bread volume. [155] It has also been reported that, on the other hand, the group of enzymes active on the pentosans and known as the pentosanases, have an anti-staling effect on baked products. [140]

2.2.4.2 Emulsifiers

Emulsifiers are a group of compounds belonging to the surface- active agents or surfactants class of molecules. They are fatty substances possessing both lipophilic and hydrophilic properties. [137] This involves the unique property of being able to suspend two substances that normally do not dissolve in each other, and in food systems this typically includes water and an oil. [136] Depending on their function in the baking process, emulsifiers can be further classified: crumb softeners including α-monoacylglycerols with a level of addition of approximately 0.5%, and the dough strengtheners particularly diacetyl tartaric acid esters of monodiglycerides (DATEM), Calcium stearoyl-2-actylate (CSL), and sodium stearoyl-2-lactylate (SSL). The last of these serves both functions in bread making. [93, 136, 137] In addition, the swelling of wheat starch granules during gelatinisation has been reported to be delayed in the presence of certain emulsifiers. [156, 157] The mechanism by which emulsifiers retard staling is based on their formation of a complex with amylose. Due to their differing abilities to form complexes with amylose, their contribution to retardation of staling also varies. [158]

The incorporation of emulsifiers into bread formulations can be used as a means to reduce the caloric value of bread by replacing some of the harder shortening materials with lower calorie emulsifiers. [159]

3

3 Literature review: II. Fourier transform infra-red analysis

3.1 FT-IR of protein secondary structure

3.1.1 FT-IR and protein analysis

FT-IR is one of the earliest experimental methods applied to the evaluation of the secondary structure of polypeptides and proteins. [160-162] Typically FT-IR spectroscopy involves varying the wavelength and monitoring the extent to which the IR radiation is absorbed by a sample. [163] Attenuated Total Reflectance (ATR) FT-IR is a type of internal reflection spectroscopy in which the sample is placed in contact with an internal reflection element (IRE) of high refractive index. Infrared radiation is focussed onto the edge of the IRE, reflected through the IRE, and then directed to a suitable detector.[164, 165] One of the advantages of using ATR/FT-IR in protein structural analysis is that the analysis can be performed on samples having a variety of forms. These may be solids, including powders, [166-168] or may also involve the use of KBr discs, as well as aqueous solutions, [160, 169, 170] organic solvents [171] and phospholipid membranes. [172, 173] The different analytical techniques are illustrated in Figure 3-1 and Figure 3-2.

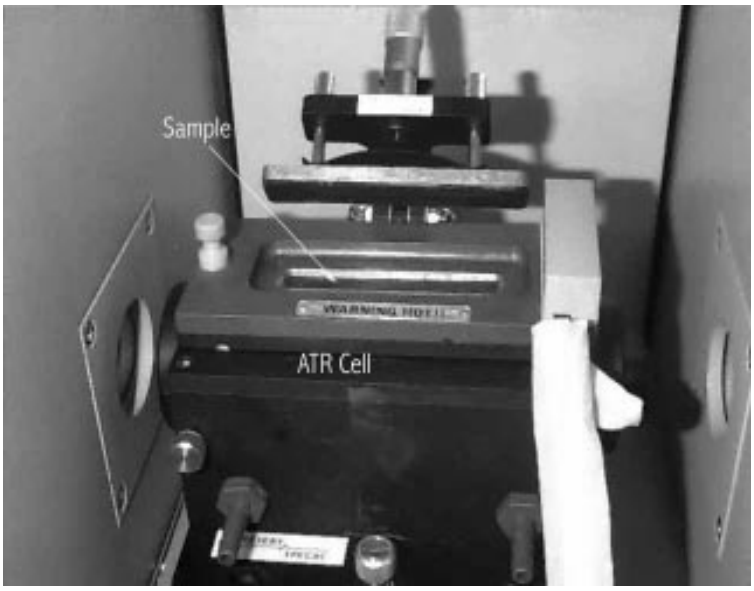

Figure 3-1 The ATR accessory used for aqueous solutions

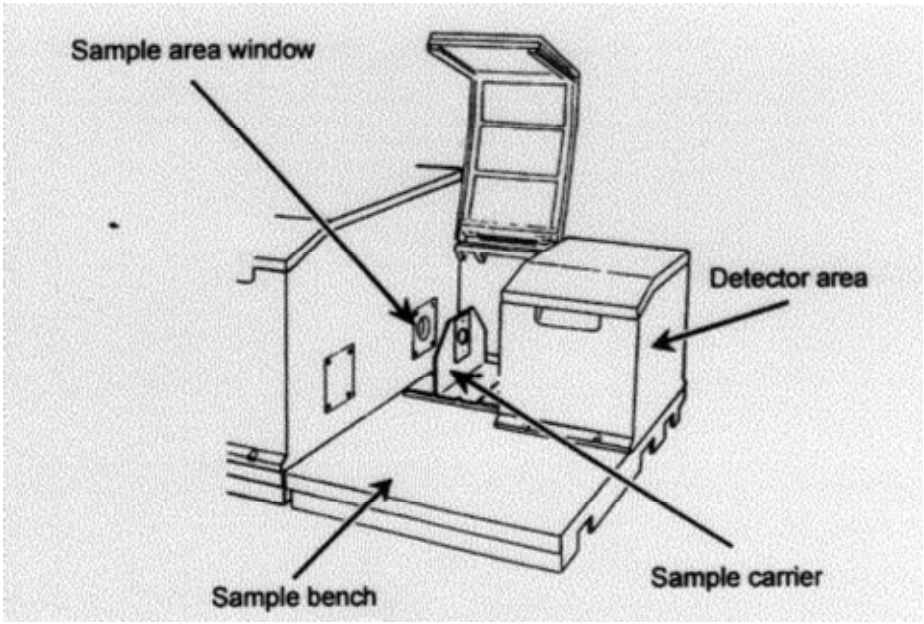

Figure 3-2 The accessory arrangement used for presentation of dry samples in the form of a KBr disc

One of the challenges encountered in using ATR/FT-IR for the structural analysis of proteins is that one of the absorption bands of water molecules is in the specific region of interest, which is referred to as the amide I region. Two techniques have been applied to solve this problem: the first is based on the use of deuterium water as solvent rather than water. [174] The second option is to use short path lengths and digital subtraction

routines. A further potential problem of ATR/FT-IR is that each particular protein is composed of a specific combination or proportion of the commonly occurring secondary structures. [175]

In recent years many factors have contributed to the revitalisation of ATR/FT-IR in protein analysis. In particular, the availability of fast computers has led to FT- processors able to rapidly compute Fourier- transforms, and further having the capability to carry out detailed normal mode analysis treatments on model biopolymers.[176] The use of stable and powerful lasers has led to the development of the Fourier transform (FT) method for IR data acquisition and reliable digital subtraction. [163] The development of methods for processing the recorded spectrum to resolve overlapping bands has also contributed to the renewed interest in the application of ATR/FT-IR in protein analysis. Examples of these methods are Fourier deconvolution, the use of second derivatives as well as band fitting algorithms. [177]

Common features of the IR spectra of proteins are the so-called amide bands, which arise from delocalised vibrations of the peptide linkage. [178] Elliot and Ambrose [161] were the first to demonstrate the existence of empirical correlations between the amide I and amide II bands of polypeptides and their conformation. [179]

Very little of the work in this field has specifically been done on wheat flour doughs for the quantification or examination of the protein secondary structure. Accordingly there is relatively little literature available. The importance of the current study has been to explore changes in protein secondary structure brought about by the addition of baking ingredients, to establish methods for quantifying protein secondary structure, and also to investigate the relationship between the dough strength and a specific type or component of protein secondary structure. The current lack of published work related to each of these important scientific problems have formed the basis for the research reported in this thesis.

3.1.2 ATR/FT-IR of proteins

A considerable amount of experimental and theoretical research has been done on the IR spectra of proteins and polypeptides [179-186] and this will be reviewed briefly here.

3.1.2.1 ATR/FT- IR of protein secondary structure in different environments

Byler and Susi [160] obtained the spectra of 21 globular proteins in deuterium oxide solution. They reported that after applying Fourier self-deconvolution the amide I band of

each protein consists of six to nine components which are helical segments, extended β-segments, unordered segments and turns. Protein secondary structure in aqueous solutions was studied by Dousseau and Pezolet [179] They reported the spectra of 13 proteins of known crystal structure and a comparison of the classical and partial least-squares methods was included.

Protein secondary structures in water were studied by Dong and co-workers. [184] Twelve globular proteins from a variety of sources were selected as having different combinations of protein secondary structure. The spectra were recorded for these proteins in aqueous solution at 20 ºC and it was reported that band frequency assignments for α-helix, β-sheet, unordered, and turn structures were highly consistent among all of the proteins studied.

3.1.2.2 Secondary structure of proteins with parallel β-chains

The secondary structure of proteins having parallel β-chains were reported by Susi and Byler. [180] The characteristic absorption bands for the parallel β-segments are observed at 1626-1639 cm^{-1} (strong) and around 1675 cm^{-1} (weak).

3.1.2.3 Secondary structure of denatured protein

Purcell and Susi [183] investigated the conformational changes induced by denaturation and reported that in the denatured form there is structure similar but not identical to, the β-structure of native proteins. This structure is characterised by absorption bands around 1615-1618 cm^{-1} and 1684-1688 cm^{-1}. Protein secondary structure changes by low-energy ion irradiation are reported by Cui and co-workers. [186] They found that irradiation decreased the proportion of α-helix and β-turn and increased random coil and β-sheet structures.

3.1.3 ATR/FT-IR of wheat flour dough systems

Changes in secondary protein structure during mixing and development of high absorption (90%) flour and water mixtures have also been examined. [187] The wheat flour dough was evaluated at various mixing times. When amide I band was deconvoluted, peaks representing those for known secondary structural features of proteins were revealed. The peak assignments included α-helix, β-turn, β-strand and β-sheet.

3.1.3.1 ATR/FT-IR of wheat flour dough

Popineau et al. [188] studied the conformations of gluten proteins selected as having differing gliadin and glutenin compositions. They studied both the conformation of whole

gluten and the subsequent fractionated preparations; in addition two different environments were used with one in solution the second was the dough mass. The results demonstrated that in the hydrated viscoelastic state, the β-sheet content, especially that of intermolecular β-sheet conformation, of gluten proteins was higher than that observed in solution. They also found that in viscoelastic gluten fractions, the proportion of intermolecular β-sheet increased as the glutenin content increased. Their band assignment was slightly different than that used by some other workers [187] in that their assignment included only intermolecular β-sheet, intramolecular β-sheet, α-helix, β-turn.
The effect of some additives on wheat gluten solubility was investigated by Mathlouthi [189] using a structural approach. The additives they used were potassium chloride, sodium chloride and cysteine in combination with partially hydrolysed gluten. The addition of potassium chloride and cysteine increased the band associated with the OH stretching mode of water at around 2800-3800 cm^{-1} and the intensity of this band was correlated with the amount of water absorbed by the protein polymer. [190] The area of the OH stretching band was shown to be proportional to the sample water content [166, 177, 190, 191] confirming the correlation previously observed by Rueegg. [192] The band assignments for amide I were intermolecular β-sheet, intramolecular β-sheet, α-helix, β-turn, and antiparallel β-sheet. These assignments are in broad agreement with most of the research reported in this field. [193]

Dough optimum mixing time was studied by Seabourn and others [194] who plotted the mixing time against the second derivative band area ratio of the peak 1336 cm^{-1} / 1242 cm^{-1}. The peaks are amide 111 peaks and the peak assignments were: 1336 cm^{-1} for α-helix and 1242 cm^{-1} for β-sheet. Samples with short mixing times tended to develop β-sheet faster than those with long mixing time. This indicates that the protein network in a dough system is dependent on the formation of specific secondary structures including the β-sheet structure.

3.1.4 ATR/FT-IR of purified wheat flour gluten

The effects of temperature and water content on the secondary structure of wheat gluten were investigated by Georget and co-workers. [195] For the study, gluten samples were conditioned at water content levels of 0%, 13 %, 47%. Over a temperature range of 25-85 ºC it was found that in the 0% hydrated sample heating the material did not affect the conformation of the protein secondary structure. On the other hand, at 13% hydration the band assigned to intra-molecular β-sheet at around 1630 cm^{-1} was shifted to a lower

frequency and became deeper compared to the band at 1613 cm^{-1} which is due to inter-molecular β-sheet. At 47% hydration both peaks were replaced by one broad peak centred at 1620 cm^{-1}.

3.1.5 Protein secondary structure of glutenin

The hydration of the glutenin fraction having high molecular subunits was studied by Belton. [196] It was reported that increasing hydration affects the conformation of the protein secondary structure in favour of a more extended structure of the β-sheet-type. The presence of the disulfide bonds was found to enhance the formation of β-sheet. In addition, the elasticity of high molecular subunits was influenced by the balance between the interactions involving protein-protein and protein-water hydrogen bonding.

3.1.6 Protein secondary structure of gliadins

The interactions between gliadins and dextrins and their effect on protein secondary structure were investigated by Secundo and Guerrieri. [197] It was reported that gliadin secondary structure changes upon heat treatment both in the presence and absence of dextrin. The changes are reflected as a decrease of the band at 1651 cm^{-1} and corresponding increases of the bands at 1628 cm^{-1} and 1690 cm^{-1}. The results also indicated the formation of hydrogen bonds between gliadin and dextrin molecules.

3.2 ATR/FT-IR studies of the enzymatic degradation of starch

3.2.1 Hydrolysis of starch by amylases

Starch is a major storage product of many economically important crops including wheat, rice, maize, tapioca, and potato.[198] The main two constituents of starch are amylose and amylopectin, with amylose having a linear structure while amylopectin is branched. Both amylose and amylopectin consist of α(1-4) linked glucose units in the linear regions, amylopectin also containing α-1-6 links at the branch points. [199] Carbohydrates owe some of their functionality to such structural features as glycosidic bonds and hydrogen bonding.[200] α-Amylase (α-1,4-glucan 4-glucanohydrolase, E.C.3.2.1.1) cleaves α-1,4-glycosidic links at random in an endo mode yielding shorter fragments. [201]

On a global basis, the starch industry uses about 30% of the enzymes produced on a commercial scale. The application of these amylases include the conversion of starch to glucose syrups and in the liquefaction process to produce dextrins. [202]

3.2.2 ATR/FT-IR of carbohydrates

The region of 1300-900 cm^{-1}, (C-C and C-O) in the IR spectrum is sensitive to the conformation of carbohydrates. [199] In addition, the region referred to as the anomeric region at 950-750 cm^{-1} is frequently used in carbohydrates analysis. This is applied in order to distinguish bands characteristic for α and β conformers corresponding to the vibrations for both monosaccharides and carbohydrates having higher degrees of polymerisation. [203]

3.2.3 ATR/FT-IR of oligosaccharides and polysaccharides

The effect of mass concentration on the structure of oligosaccharides and their constituent monosaccharides has been studied by Mathlouthi.and co-workers. [200] A series of oligosaccharides were employed in which the position of the glycosidic bond differed. In order to distinguish between the oligosaccharides and their monomers time dependent spectral measurements were undertaken during acid hydrolysis of some of the oligosaccharides. It was concluded that the bands most affected are those situated in the region 993-966 cm^{-1} which is due to the stretching of the C-O linkages within the C-O-C structure which is the glycosidic linkage. These researchers achieved the tentative assignment of a very large number of the observed IR bands.

The glycosidic linkage in oligosaccharides has also been investigated by Sekkal et al. [204] who studied eight oligosaccharides, the results of this study provided confirmation of the assignments of Mathlouthi. [200] In a further study in this field, the influence of hydration of food additive polysaccharides has been reported [205] indicating that it is possible to distinguish sugars and polysaccharides by ATR/FT-IR with no apparent loss of information in hydrated samples.

3.2.4 The use of ATR/FT-IR in the study of starch structure

Starch granules exhibit a crystalline-amorphous structure to varying extents. [206] In the native form of starch, amylose and amylopectin molecules are organised in granules as alternating semi-crystalline and amorphous layers that form growth rings. [207]

Several researchers have used ATR/FT-IR in structural analysis of starch. [206, 208-215] In a study of the short range structure by van Soest [215] the IR spectrum in the 1300-800 cm^{-1} region was utilised corresponding with C-C and C-O stretching modes of vibration. It was reported that the peak at 1047 cm^{-1} is characteristic of crystalline starch and the peak at 1022 cm^{-1} is characteristic of amorphous material. Additionally, the peak at 994 cm^{-1}, which is related to intramolecular hydrogen bonding of the hydroxyl group at C-6 was reported to be sensitive to the level of water present.

ATR/FT-IR was also used to study the organisation of the external region of the starch granule by Sevenou. [206] For this study, different types of native starches were used and compared to acid hydrolysed and gelatinsed starch, however, ATR/FT-IR was not able to differentiate between A-and B-type crystallinity. The results showed that starches from potato and amylomaize were more ordered on their outer regions, which explains their greater resistance to enzymatic hydrolysis.

FT-IR has also been used to study the granular structure of resistant starches having the type 4 characteristics. [213].For this, starches including normal, high amylose as well as waxy corn starch citrate were investigated. The intensity ratio of 1016 cm^{-1} /1045 cm^{-1} was used to estimate the ratio of amorphous to crystalline phase in the starch citrate. These are the same two peaks used by van Soest to characterise the amorphous and crystal structure with a shift in the band position.

3.2.5 ATR/FT-IR of starch gels

Gelation of starch starts with the penetration of water into the amorphous part of starch causing the granule to swell. [207, 216]The endothermic water uptake changes the crystal structure to that of a gel with the temperature depending upon the type of starch and its source. [201] This is illustrated in Figure 3-3.

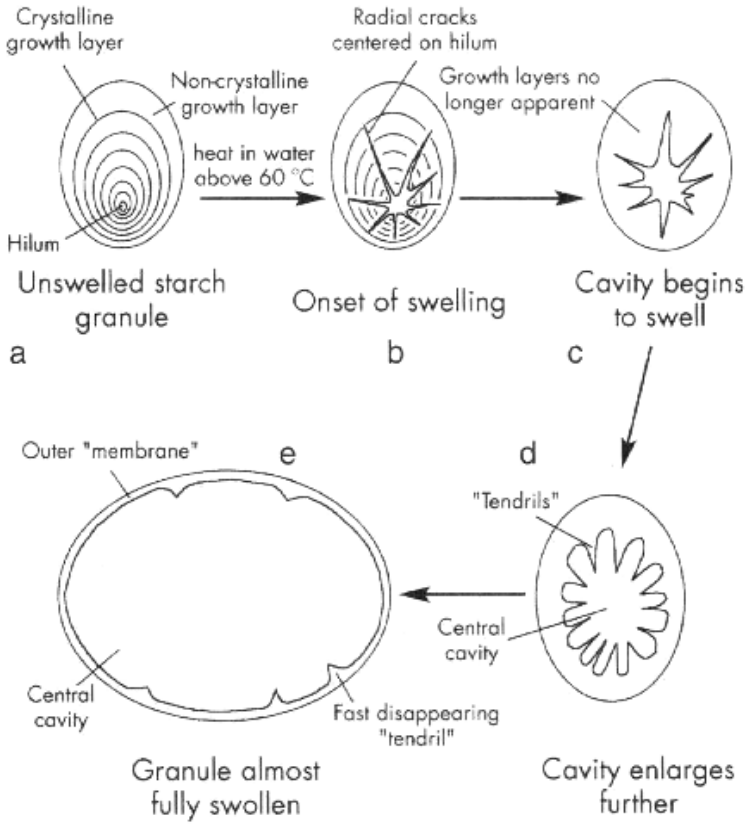

Figure 3-3 Starch gelatinisation process [207]

From the results of a study on the pressure- induced gelation of selected starches, Rubens and co-workers [216] proposed a two step mechanism. First the hydration of the amorphous regions leads to the swelling of the granule and distortion of the crystalline regions which leads to the destruction of the granular structure. In the second step the crystalline regions become more accessible for water. They reported that, during gelation, increasing the pressure results in an increase in the relative proportion of the amorphous compared to the crystalline structure.

Wheat starch gels were investigated by Wilson and Belton. [217] They monitored the behaviour of starch gels over a period of 21 days following gelatinisation. During the storage of a wheat starch gel in a sealed cell, no changes were observed, but when the samples were exposed to an environment of gradual drying, the peak representing the starch having crystalline structure increased until it returned to its original intensity. It has also been shown that the kinetics of starch retrogradation can be monitored by following the changes of the peak at 1046 cm^{-1}. [217]

Goodfellow [199] investigated the gelation and retrogradation in amylose and amylopectin and found that they show a fast initial change, amylopectin also showed a second slower change over a time scale of approximately 400 hours. The fast changes in amylose can be summarised into the increase in intensity of the band at 1053 cm^{-1}, while in amylopectin the increase in the intensity of the band at 1053 cm^{-1} is much greater. The band at 1053 cm^{-1} is correlated with starch recrystallisation and retogradation.

In a study of the retrogradation and physical ageing of model starch systems Smits and co-workers [218] distinguished between the physical aging which takes place below the glass transition of starch and retrogradation which takes place above the glass transition of starch. The changes reflected changes in the ratios of the peak intensities at 1047 cm^{-1} (crystal) and 1022 cm^{-1} (amorphous) during storage, as described in Figure 3-4.

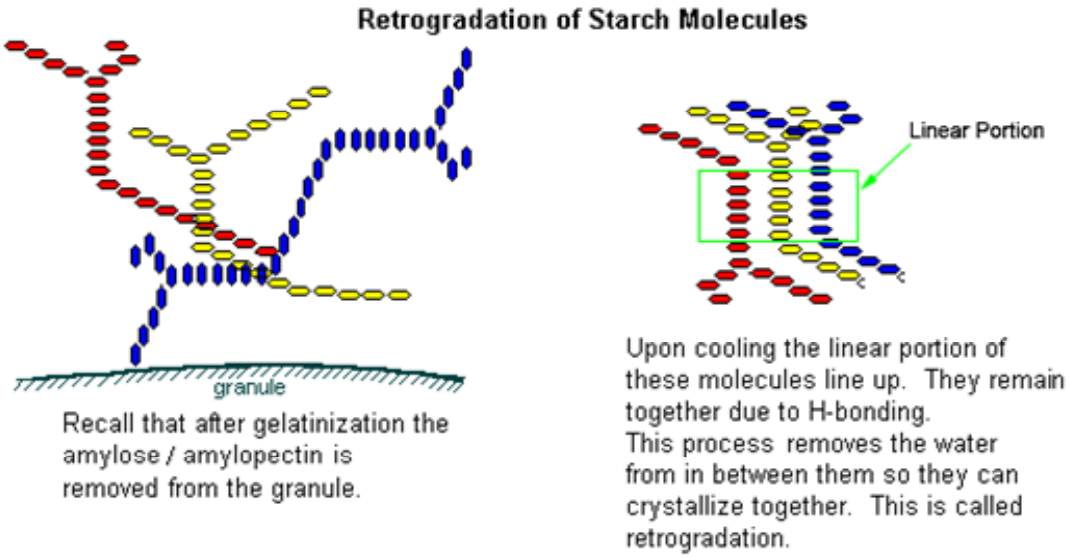

Figure 3-4 The stages in the process of starch retrogradation [219]

Summary of significance of project and statement of aims

This investigation has been developed on the basis of the following:

- Wheat is a major crop and food source internationally;
- In the processing of wheat, milling is generally involved and the resultant meal (flour) is mixed with water to form a dough;
- In the commercial utilisation of wheat quality control is required in order to ensure that the flour being produced is suited to the processing parameters selected or available so that the quality of the resultant foods meets the expectation of consumers;
- Among the specific measurements required so that quality can be readily measured and thereby controlled, the incorporation of enzymes and the use of food additive ingredients are ways in which processing can be manipulated for enhancement of quality;
- The use of flour also relies upon the extent to which starch is mechanically damaged and the susceptibility to enzymatic hydrolysis as well as retrogradation;
- One of the current constraints is the availability of rapid, reliable analytical procedures that allow for the rapid analysis of relevant parameters during milling and flour processing, in particular the lack of non-destructive techniques is a limitation upon the implementation of reliable quality control approaches at a commercial level;
- At a research level there remains a need to access techniques which facilitate the evaluation of protein structure during dough development;
- Whilst infra-red reflectance and transmittance technology has been adopted at a variety of points within research and industrial situations for grain processing and utilisation, much remains to be elucidated in these areas; and
- One recent technological development involves the use of new generations of infra-red instrumentation. In conjunction with deconvolution software there is considerable scope for rapid developments in these areas with strong implications for our understanding of grain processing as well as potential for practical application.

It is within this context that the following aims have been developed for this investigation.

Statement of hypothesis and aims

The hypothesis of this project has been that the use of deconvolution software and infra-red spectroscopy will allow the monitoring of a range of processing and chemical changes in wheat flour doughs.

The primary aim of the project has been to evaluate this hypothesis and the specifc objectives included:

1. To study the use of the current generation of instrumentation and software as a tool for monitoring changes during the stages of bread making;
2. To apply the techniques to a study of the influence of enzymes, oxidants and reductants during dough development and breadmaking;
3. To evaluate the changes occurring in the secondary a structure of the protein components of dough; and
4. To assess the influences of the enzyme α- amylase on starch properties and staling of the resultant baked products from bread making.

4

4 Material and Methods

4.1 Materials

4.1.1 Wheat flour

Wheat flour was obtained from Manildra Group Australia, from milling of Australian hard wheat varieties, suitable for bread making. The flour properties were: Protein 11.8 %, Moisture 13.3%, Water Absorption 66.0 %, Extensograph Height 45 Minutes (min) = 400 Brabender units (BU), Extensograph Length 45min= 20.5 centimetres (cm), Falling Number =500 Seconds (s) and Dough Development Time= 5 min.

4.1.2 Baking ingredients

The materials and suppliers are show in Table 4-1

Table 4-1 Materials and suppliers of bakery ingredients

Supplier	**Material**
CSR, Australia	White sugar
Saxa, Australia	Table salt
Crisco Australia	Blended vegetable oil
BDH Laboratory Supplies, England	L-ascorbic acid
Lowan instant dry yeast, Australia	High activity, instant dried yeast
Aldrich Chemical Company	Fumaric acid
BDH Laboratory Supplies, England	L-cysteine
Sigma	α-Amylase (from *Bacillus sp*, product code A4551)
Merck	L-cystine, L-Cysteine HCl
Sigma	Dextrin type 1, dextrin type 11, and D-glucose

4.2 Instruments

4.2.1 ATR/FT-IR and KBr disc

FT-IR spectra on the dough were recorded using a Perkin-Elmer 2000 ATR/FT-IR spectrometer equipped with ATR accessory 45° angles ZnSe crystal. For all analyses, except for L-cysteine and L-cystine, the KBr Disc accessory was used. For this, the dry samples were mixed with potassium bromide in a ratio of 1: 100, ground to a very fine powder and pressed to make a disc.

For all spectra using ATR and the KBr disc accessories, 100 scans were taken at an interval of 1 cm^{-1} and recorded at a 4 cm^{-1} resolution to give an optimal signal to noise ratio, generally in the wave number range of 700 cm^{-1} -4000 cm^{-1}. In the case of the dough and for the KBr disc the range was from 400 cm^{-1} -4000 cm^{-1}. The background spectrum was obtained using the empty crystal, and before each measurement the crystal was washed with water, acetone and dried with nitrogen.

4.2.2 Dough mixing, bread making and assessment instruments

For bread texture measurement the Texture analyser from Stable Micro Systems, England model TA-XT2 was used. Dough mixing for spectroscopy experiments was carried out in a small Kenwood kitchen mixer. The balance used was Mettler Toledo PB303DR and for pH measurements, a Hanna Instruments, Italy model pH211 pH meter was used. For bread making, a Panasonic bread maker from Panasonic Australia Pty. Ltd. Australia SD-253 was used. For colour measurement Minolta chromameter CR-200 was used.

4.3 Methods

4.3.1 Bread making

In the preparation of bread loaves for the investigation of the impact of different additives, the basic baking ingredients used were wheat flour, sugar, salt, water, yeast and shortening. L- ascorbic acid, L-cysteine, fumaric acid, α-amylase as well as sodium chloride were added in varying amounts in individual experiments to study the effect of addition of each ingredient. Loaves were prepared following a method based upon that described by the Bread Research Institute of Australia (BRIA) (1989). The ingredient formulation used is shown in Table 4-2

Table 4-2 Ingredient formulation used in baking

Ingredient	Proportion (% based on flour weight)	Amount (g/450g flour)
Flour	100	450
Water	66	297
Sugar	2	9
Shortening	2	9
Salt	1.8	8.1
Yeast	1.5	6.8

Using the Panasonic bread maker, a rapid cycle of 1 hour (h) and 55 min was selected for preparing the baked loaves. Immediately following completion of the cycle, the baked loaves were removed from the baking pans and placed onto wire racks. Loaves were then allowed to cool at room temperature for 1 h. Slices were cut after the completion of the cooling period. Slices were packaged in sealed plastic bags to prevent any moisture loses. The texture was measured, and the slices were labelled with the date and the texture was further measured on samples stored for 2, 4, and 6 days.

4.3.2 Bread texture measurements

Texture measurements were carried out by the standard procedure (AACC 2000) of the American Association of Cereal Chemists (AACC) using the Texture Analyser (Model TA-XT2, Stable Micro Systems, England). The instrument was operated using a computer loaded with Texture Expert for Windows software, © 1995, supplied by Stable Micro Systems. The settings and parameters used were as shown in Table 4-3.

Table 4-3 Settings employed with the texture analyser

Parameter	Setting used
Mode	Measure Force in Compression
Option	Return to Start
Pre-Test Speed:	1.0 mm/s
Test Speed:	1.7 mm/s (i.e. 100mm/min)
Post-Test Speed	10.0 mm/s
Strain	40%
Trigger Type	Auto – 5g
Data Acquisition Rate	250pps
Accessory	AACC 36mm cylinder probe
Radius	(P/36R)
Load cell	5kg

All samples were packaged in sealed plastic bags until the day of the compression test, to minimise any sample drying or moisture losses. When slicing the bread care was taken to achieve the same thickness for all slices to minimise the measurement error. All samples were sliced mechanically to a slice thickness of 25mm. The probe compresses the sample slice until it has compressed 40% of the product height. It then withdraws from the sample and returns to its starting position. A graphical plot is generated illustrating the variation of force as a function of time (equivalent to the distance travelled by the probe). The samples were compressed in five different points (one in the centre and four near the corners), and average force was calculated. The software analyses the curve and tabulates specified measurements showing the characteristics of crumb firmness.

Firmness is defined in this method as the force required compressing the product by a preset distance (i.e. force taken at 25% compression of 25mm) (AACC, 2000) A 25% compression of 25mm thick sample = 6.25mm compression distance at which point the compression force value (CFV) was taken. Whilst force can be expressed in units of grams, kilograms or Newtons (AACC, 2000), in the current study the unit used was the Newton (abbreviated N).

4.3.3 Measurement of loaf volume

Bread volume was measured by using the Royal Australia Chemical Institute (RACI) standard procedure (RACI 1995). This method measures bread loaf volume as estimation

from the sum of the two circumference values of the loaf. The second measurement was taken perpendicular to the first. The measurements were taken after one hour of cooling at room temperature and the final value used is the sum expressed in centimetres.

4.3.4 Dough preparation for spectroscopy experiments

4.3.4.1 Dough for protein secondary structure studies

Distilled water (66 ml) was added to 100 g of flour in the Kenwood mixer then the appropriate volume of ascorbic acid or L- cysteine solution was added to the flour and adjusted to give final water addition of 66 ml. The dough was mixed for 8 min.
A small piece of the dough was cut using scissors and loaded onto the ATR accessory immediately. The remainder of the dough was covered with plastic and rested for 20 min in the mixing bowl and loaded onto the ATR accessory as soon as 20 min had elapsed.

4.3.4.2 Dough preparation for the enzymatic degradation of starch

Flour (100 g) was added to 66 ml of water and mixed in a beaker using a spatula, α-amylase was added and the dough re-mixed before loading on to the ATR crystal. There was a delay of two minutes from the time of addition of the enzyme until the loading of the dough onto the crystal the latter is considered as zero minutes. The remainder of the dough was incubated at 35 ºC and every 5 minutes a spectrum was collected.

4.3.5 Water subtraction methods

4.3.5.1 Water subtraction for protein secondary structure studies

The band which interferes with Amide I absorption band is that occurring at around 1640 cm^{-1} and this arises from the water bending vibration mode. In order to elucidate any information from the amide I band, the contribution of absorption of this band has to be removed from the dough spectra or using deuterium oxide solution as solvent, instead of water, to shift the absorption band due to water bending to a region out of the amide I region. Deuterium oxide is usually employed instead of water because of its greater transparency in the region of interest. [220]

In this study the band occurring due to the combination of bending and libration mode of vibration of water molecular appear at around 2125 cm^{-1} was used to subtract the water

from the dough spectra assuming according to the approach described by Dousseau. [221] The complete removal of the band around 2125 cm^{-1} to give a flat base line will also remove the band at 1640 cm^{1} leaving only the absorption band in the amide I region which is due to the amide bond vibration mode.

4.3.5.2 Water subtraction for enzymatic degradation of starch

No water subtraction procedure was required for those samples where the FT-IR KBr disc analysis was utilised. This was because there is no water involved (as is sample). Also no water subtraction procedure was needed for the enzymatic degradation of starch experiments because the water absorption band does not interfere with the regions of interest, corresponding to 800- 1300 cm^{-1} (C-C, C-O).

4.3.6 Deconvolution

4.3.6.1 Deconvolution for protein secondary structure studies

Deconvolution is a resolution- enhancement procedure used to resolve overlapped and hidden peaks. Its application to IR has made more detailed studies of globular proteins possible. [222] In this study, deconvolution was carried out by assuming an initial Gaussian line-shape function for all the peaks. [223] In order to measure the relative areas of the partially resolved amide I and II band components, the deconvoluted spectra were curve fitted. Peak fit 4.21 software (Hearne Scientific Software) was used with the following settings: 63 filter and 9.57 and 0.05. The base line perfectly matching the section (880 - 2580 $cm^{-1)}$ was found to be a non -parametric base line with variance r^2 = 0.9992. The data very closely matched the generated spectra. All the data for all the experiments were treated in the same way. Dividing the sum of the areas of all components associated with given conformation by the total amide I or II band gives the relative area (%) which was used for the data analysis in this study, using the approach widely used by other researchers. [224]

4.3.6.2 Deconvolution for the enzymatic degradation of starch studies

All spectra were deconvoluted using peak fit software (4.21) A section for the region from 900 cm^{-1} to 1200 cm^{-1} was base lined adjusted and peak fitted assuming Gaussian shape for the peaks. The sum of the area of the peaks in the region of 900 cm^{-1} -1200 cm^{-1} was

taken and the relative area of the individual peaks were calculated. The same settings were used for all spectra.

4.3.7 Solutions preparation

4.3.7.1 Preparation of solutions for studies of protein secondary structure

L-Ascorbic acid or L-cysteine HCl (0.1g) was dissolved in 100 ml of distilled water in volumetric flasks and sonicated for 10 min in a sonic water bath to facilitate dissolution. Volumes equivalent to the following dosages of ascorbic acid were added to individual flour samples: 0.0, 50, 100, 150, 200, 250, 300 ppm and for L-cysteine the following dosages were added 0.0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0 ppm.

4.3.7.2 Solution preparation for the enzymatic degradation of starch studies

The spectra of Dextrin type1, Dextrin type 11, D-glucose and unmodified starch were obtained by dissolving 2 g of each in 5 ml of distilled water.

5

5 Results and discussion: the interaction of ingredients during baking

5.1 Introduction

Bread is widely regarded as the most important staple food in the western world. [130] There is a great variety of baking processes and the differing baking performance of flours has led to the term baking quality being difficult to clearly define. In addition this complexity is compounded if we seek to establish general criteria of additives effects. [131] For example it has been reported that, on a worldwide basis, more than 60 distinguishable types of flat bread are made. [225] Over time there have been many developments in bread making and today additives and technological aids are extensively used in the baking industry. [226] One of the significant changes has been in the increasing use of enzymes in bread formulations over recent decades. [227] Enzymes as technological aids are usually added to flour during the mixing step of the bread making process. [228] However it is significant that grains naturally contain a large number of enzymes and the variation in their activity levels may exert strong effects on the quality of cereal raw materials and the resultant food products. [144]

5.2 Effect of sodium chloride

5.2.1 Bread volume

A significant increase in bread volume was found when salt dosage increased from 0.0% to 1.6% as shown in Figure 5-1, this is due firstly to the function of salt in bread making process. Additionally, there are effects of salt on the function of various components and molecules incorporated during the bread making process including the α-amylase routinely added to dough formulations. The results confirm the findings of other researchers: Salt is known to control yeast growth, at 0% salt concentration the yeast

fermented the dough too quickly producing gases in the dough system such that this did not coincide with the complete development of the gluten network. As a result the gases formed were not fully trapped and some of the gas burst through the dough surface and escaped without contributing to the bread volume. The inflation of the dough was incomplete because the rate of the production of these gases was higher than the dough gas retention capacity. In order to produce a loaf of bread with a light and even crumb texture, the dough must be able to retain the gases produced by the yeast fermentation as discrete gas cells for a sufficiently long period. [229] In the current study, the observation was made that some gas bubbles holes appeared on the surface of the bread crust for loaves with 0 % concentration of salt. This is consistent with a rupturing of the matrix during baking, leading to the establishment of continuous gas phase and consequently, the rapid loss of gas. [229] There was probably also a decrease in bread volume due to the effect of salt on α-amylase activity. As salt increases the energy necessary for chemical and physical reactions involving water [230], so the addition of sodium chloride regulates the rate of starch hydrolysis and sugar production by α-amylase. This, in turn regulates yeast growth and gas production rate, resulting in moderate inflation and a desirable final bread volume.

Additionally the effect of salt on dough rheological characteristics cannot be ignored, as salt strengthens dough structure as reported by other researchers. [231] Salt effectively toughens the gluten. [232] Accordingly, the absence of salt weakens the dough, causing the structure to be unable to hold and trap the gas, leading to the loaf having a small volume bread. On the other hand the presence of salt contributes to the structure of the dough and the gluten network producing strong dough capable of retaining the gases produced by the yeast.

Increasing the salt in the dough system beyond 1.6% results in a considerable decrease in bread volume. Increasing salt increase α-amylase hydrolysis reaction activation energy and lower the water activity. [233-235] resulting in less sugars produced by α-amylase for yeast to produce carbon dioxide in dough system and increase the volume, also sodium chloride inhibit the growth of yeast and affect the amount of gases in the dough, the increase in salt dosage in the dough system produce strong dough structure hard to be inflated by the low rate of gases produced by the inhibited yeast.

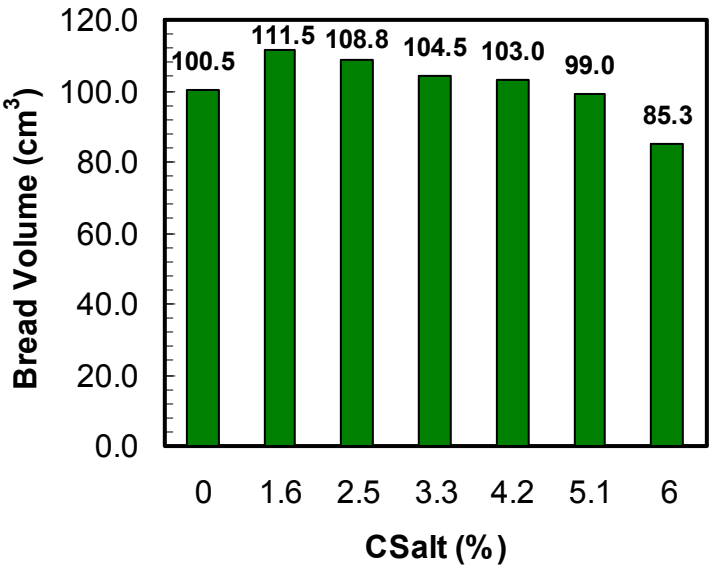

Figure 5-1 The effect of sodium chloride concentration on bread volume

5.2.2 Crust color

The increase in salt dosage increased crust color darkness, as shown in Figure 5-2 Minolta chromameter defines colour numerically in terms of lightness or L* value, (0 = black, 100 = white), a* value (greenness 0 to –100, redness 0 to +100) and b* value (blueness 0 to -100, yellowness 0 to +100) Minolta chroma meter values for L, a, b were recorded and (L-b) values were calculated. Additionally L-√ (a^2) –b was calculated and these values were used as an indication of the crust darkness. [236, 237] The observations of an increase in crust darkness with increasing in salt incorporation are at least partly due the inhibitory effect that salt exerts on the yeast: The salt attracts water away from the yeast surface thereby reducing their ability to grow and divide. This then impacts the level of simple sugars in the dough, including those directly added as well as those produced by α-amylase in the dough system. In comparison with the doughs containing lower levels of salt, the accumulation of sugars facilitates reaction with amino acids when, during baking, heat is applied. This produces the dark brown colour through Maillard reactions which are a series non-enzymatic reactions leading to the formation of brown products. [238]

In contrast, for samples with 0% salt the colour was relatively pale and the appearance was much whiter than for the samples with increasing salt dosage. For Maillard reactions to proceed, the minimum requirements are the presence of an unionised amino

compound, usually in the form of a protein or amino acid component, a reducing sugar and some water. [239]

The amylases promote yeast action during fermentation by degrading damaged starch into smaller dextrins thereby increasing the availability of reducing sugar groups. [240] Surface colour depends both on the physico-chemical characteristics of the raw dough particularly water content, pH, reducing sugar levels and amino acid content. [241] Values for L-b were used as a measure for crust darkness in comparison with calculated values for the colour index [L-√(a²) –b], as both of these have been suggested as useful single number parameters that may be easier to utilise than the three separate values of L, a and b measured on the crust samples. The differences in these calculated parameters were greater when salt levels were varied and as a result the wide variations were found to be useful in assessing the impact on crust darkness. The differences observed in the colour index value were higher than for L-b so the colour index appears to be the most useful in evaluating crust darkness. [236]

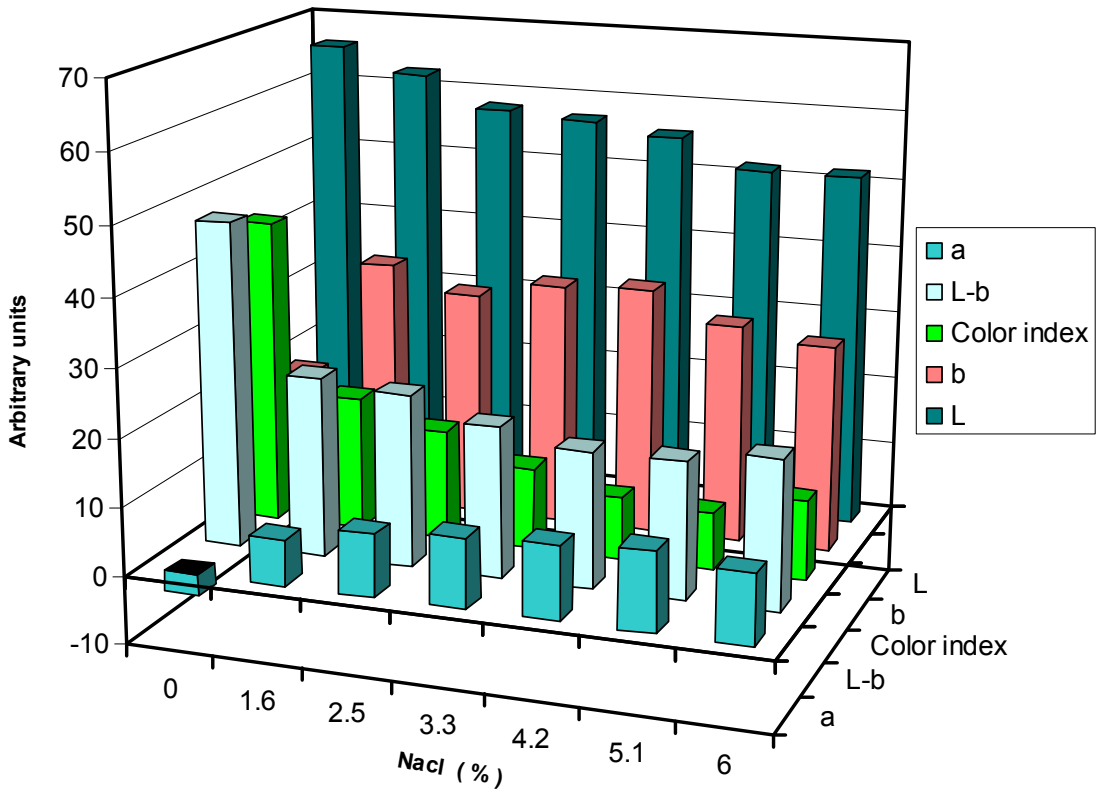

Figure 5-2 The effect of sodium chloride on crust color

5.2.3 Crumb texture

The increase in salt dosage increased crumb firmness, as shown in Figure 5-3. The increase in salt dosage affects yeast growth and so gas production rate which will then directly affect the crumb texture and structure. This reflects the fact that gas is important as it inflates the dough and enhances the texture. Increasing the salt increase the dough strength[233] and so produces a more rigid and toughened gluten network which contributes to the gas retention. The diffusion of gas through the dough phase is retarded [229] and the result is a dense crumb. Also increasing the salt dosage increases the ratio of salt to the initial dough component, this contribute to the crumb firmness.

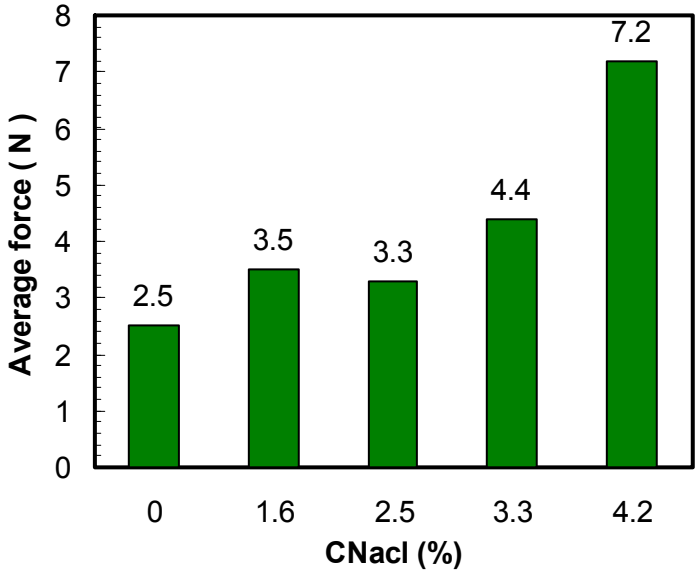

Figure 5-3 The effect of salt concentration on crumb texture

5.3 L-cysteine

Cysteine is an amino acid containing a sulfhydryl group that is available and accessible for reaction. This molecule occurs naturally in gluten proteins of wheat flour and can also be added to the dough as a reducing agent to increase dough extensibility. This occurs through its action on the gluten during mixing process of bread making. Effectively it may serve the same function as the energy of the mixer and also of proteases and these are opposite to the effects of oxidizing agents. [242] it is now well recognised that the sulfhydryl group plays an important role in dough chemistry. This occurs because sulfhydryl groups are potentially capable of undergoing a disulfide-sulfhydryl interchange

that involve the cleave and reformation of disulfide bonds. This can be mediated by the sulfhydryl groups naturally occurring in flour or by the relatively small amounts of sulfhydryl compounds added as improver additives and the reactions are referred to as disulfide interchange. [243] The reactions are shown here with R and R' representing the two gluten molecules and with cysteine as the reducing agent:

R–S–S–R' + cys–SH → R–S–S–cys + R'–SH

R–S–S–cys + cys–SH → cys–S–S–cys + R–SH

If these reactions occur, the effect is to reduce the number of cross-links between the gluten subunits and this will be proportional to the amount of cysteine added into the dough system. [242] The physico-chemical and functional properties of wheat protein are primarily attributed to the disulfide cross-links which play a significant role in the gluten net work formation in dough. [244] In addition, the effect of amino acids on bread making are important not only to the browning of the crust and its colour but also to flavour. [245] Crust browning is essential for the production of attractive bread and it has been hypothesised that crust browning is due to Maillard reactions, rather than caramelisation or pyrolysis of sugar and starch. [246]

5.3.1 Effect of L-Cysteine on bread volume

The addition of L-cysteine to the bread formula increased bread volume as shown in Figure 5-4. The added L-cysteine contributes to breaking of disulfide links during mixing and partially depolymerises the gluten proteins. [247] This results in a shorter mixing time, reduced resistance to extension [248] and a softer dough which can be readily inflated by carbon dioxide produced by yeast, giving a higher volume. At higher levels of L-cysteine addition decreased bread volumes were observed. This is due to the excessive breakage of the disulfide bonds resulting in very soft dough incapable of retaining the gas produced by yeast. [248]

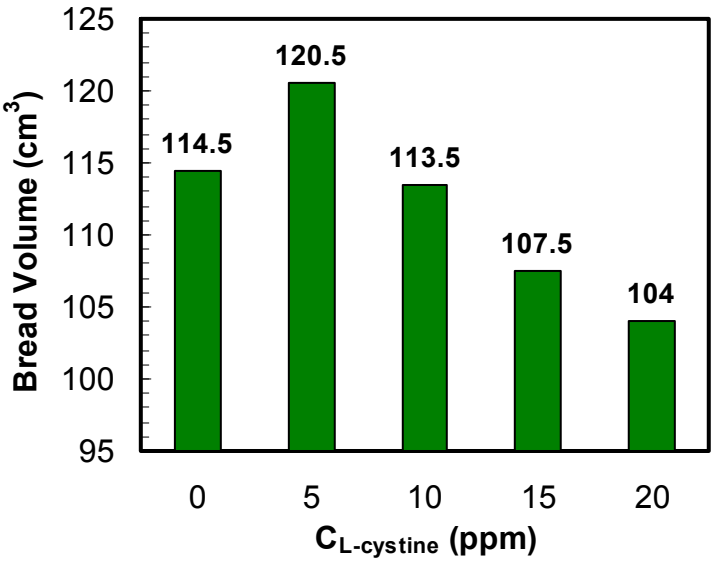

Figure 5-4 The effect of L-cysteine incorporation on bread volume

5.3.2 Effect of L-Cysteine on bread texture

Disulfide bond formation and hydrophobic interaction play a key role in protein cross-linking and thus in the development of textural characteristics. [249] In the current study, it was consistently observed that the addition of L-cysteine decreased crumb softness (Figure 5-5). The average force values increased as the L-cysteine dosage was increased. The reduction of bread softness was attributed to the small volume of the bread loaves which increased the crumb density, which is seen as a firmer crumb texture. [250]

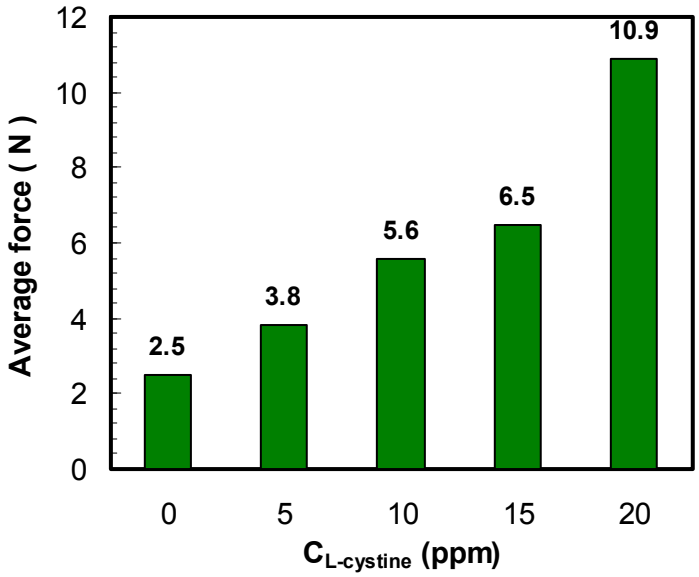

Figure 5-5 The effect of L-Cysteine addition on bread texture

5.3.3 Effect of L-cysteine on crust color

L-Cysteine addition decreased the L value for bread crust color as shown in Figure 5-6. This is due to the colour of peptides and gluten and to the occurrence of Maillard reactions involving the free amino acids. [245] L-Cysteine, as an amino acid is able to participate in Maillard reactions under suitable conditions and then contribute to the brown crust color.

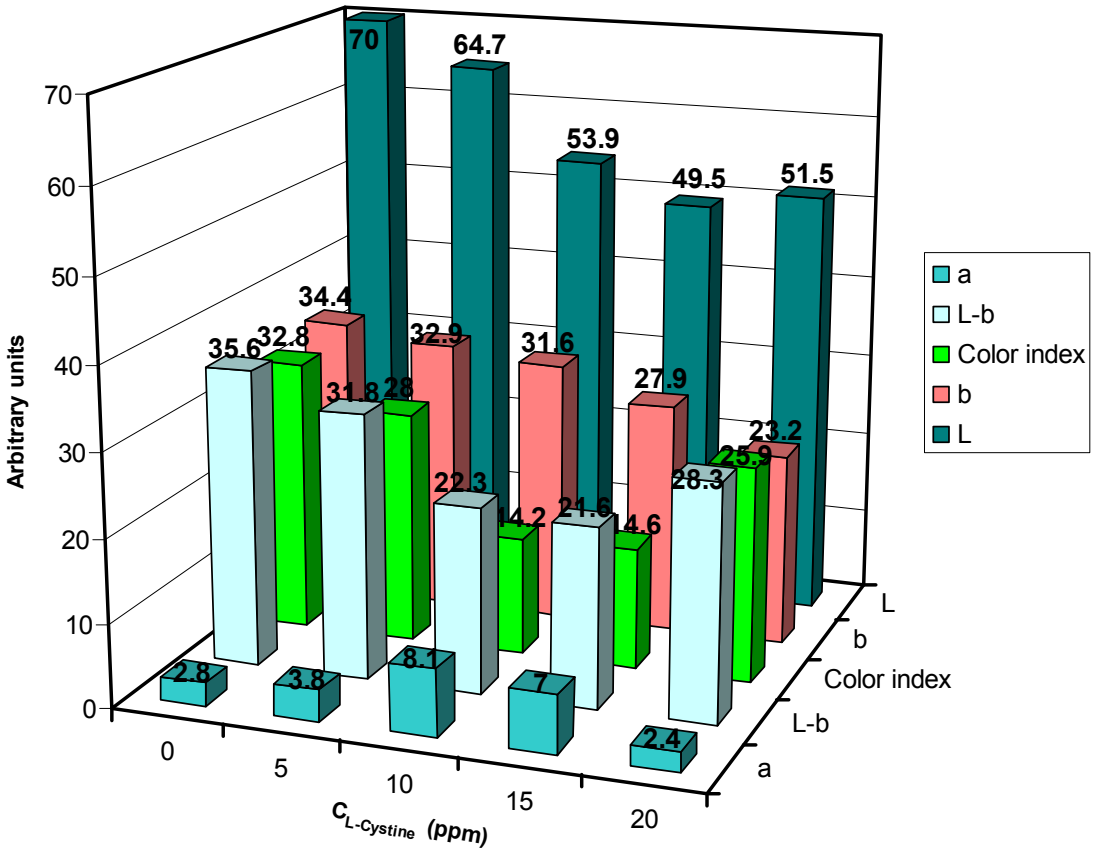

Figure 5-6 The effect of L-cysteine addition on crust color

5.4 Ascorbic acid

It has been established that the beneficial effect of ascorbic acid (AsA) in bread making is due to the oxidised form of AsA which is dehydro ascorbic acid (DHA). It has also been shown that flour contains DHA acid reductase which catalyses the oxidation of AsA to DHA. [251]

5.4.1 Effect of ascorbic acid on bread volume

The results obtained for bread volume when AsA was added to the bread formulation are presented in Figure 5-7 It was found that for each of the levels of addition from 50 to 200 ppm loaf volumes were higher than for the blank sample. AsA strengthens the dough and the gluten structure and the occurrence of sulfhydryl- disulfide interchange reaction also is used to explain the action of ascorbic acid. [252]

In addition it is likely that AsA inhibits the formation of dityrosine molecules in the dough. [253] The strength caused by the formation of disulfide bonds in the dough due to the action of AsA, makes the dough and the gluten network strong and capable of effectively retaining the gas produced by the yeast. The result is increased loaf volumes, due to the greatly enhanced gas retention capacity of the dough.

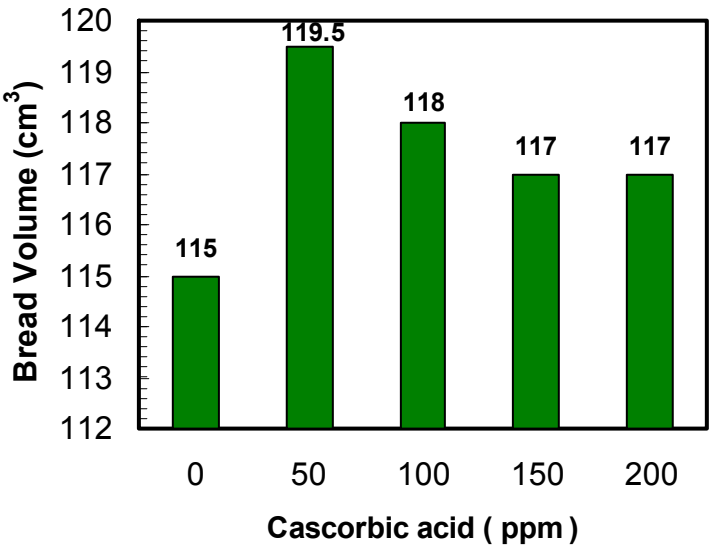

Figure 5-7 Effect of ascorbic acid on bread volume

5.4.2 Effect of ascorbic acid on bread texture

Incorporation of AsA into the bread formula decreases bread firmness (Figure 5-8). Ascorbic acid increases bread volume, resulting in low specific volume: the crumb of the bread is inflated by gas as it is produced by the yeast. The subsequent retention is facilitated by the action of AsA on the dough structure, as this is finer and lighter in texture and much less dense compared to the control samples. It has also been suggested that AsA serves a protective function for certain of the lipid components which are known to have a strong influence on loaf volume and bread quality. [254]

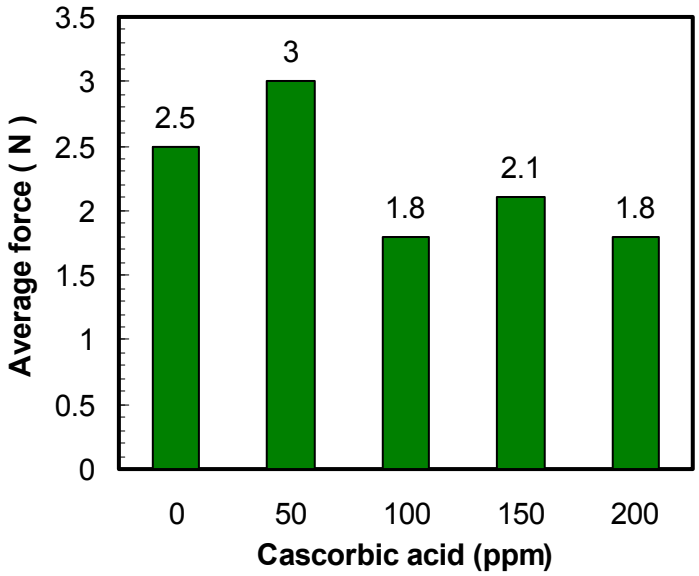

Figure 5-8 The effect of AsA on bread texture

5.4.3 Effect of ascorbic acid on crust color

The addition of AsA also influences the colour of the bread crust (Figure 5-9).

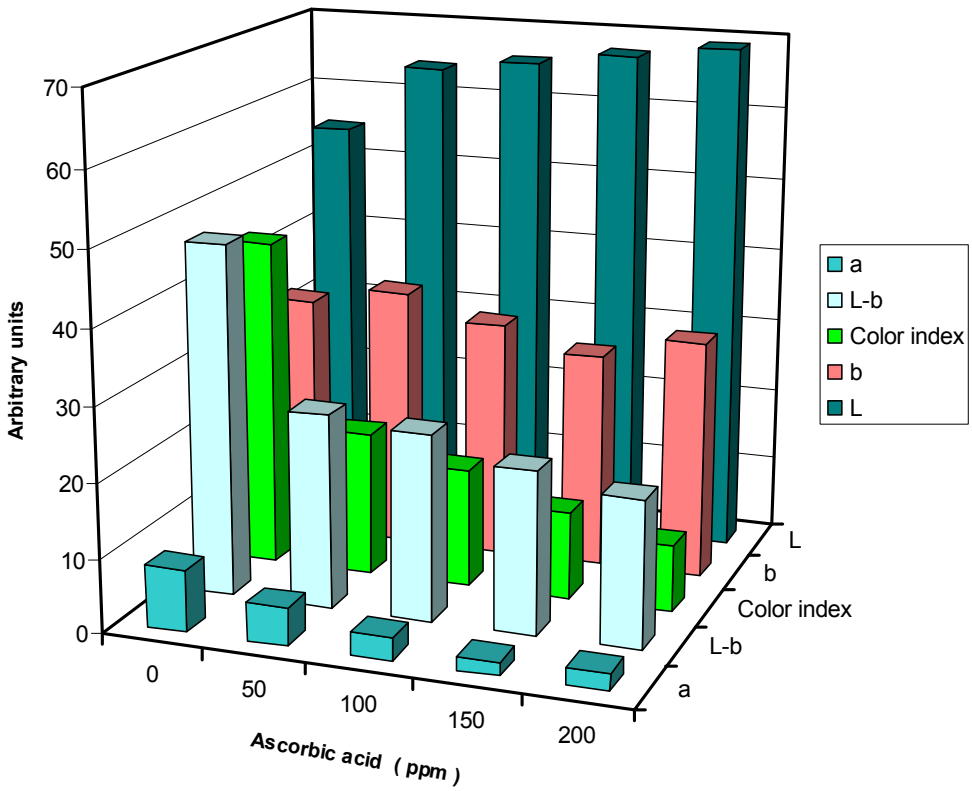

Figure 5-9 The effect of AsA on crust color

5.5 The incorporation of fumaric acid into bread formulations

Fumaric acid is a dicarboxylic, naturally occurring, organic, general purpose food acid. [255] Owing to its double bond and two carboxylic groups, fumaric acid has been widely applied industrially as a good substrate for chemical syntheses, such as esterifications and polymerisation. [256] It made synthetically by the isomerisation of maleic acid, and it can also be produced by fermentation of glucose or molasses with the mould of *Rhizopus* spp. [256, 257] As an approved additive it has potential applications in bakery formulations.

5.5.1 Effect of fumaric acid on bread volume

Fumaric acid is like other α, β-unsaturated carbonyl compounds (maleic acid and ferulic acid) in its effect on dough mixing characteristics, which is similar to the effect of cysteine and the SH-blocking reagent N-ethylmaleimide (NEMI). [258] Fumaric acid shows a reducing effect due to the double bond configuration in its molecular structure. [259] It contributes to dough relaxation by breaking the disulfide bonds between protein molecules and by this mechanism it enhances the machinability of the dough. [260] Its effect on wheat flour dough protein is illustrated in Figure 5-10, its effect on bread volume is shown in Figure 5-11, and its effect on bread texture in Figure 5-12.

Figure 5-10 The effect of fumaric acid on wheat flour dough. [260]

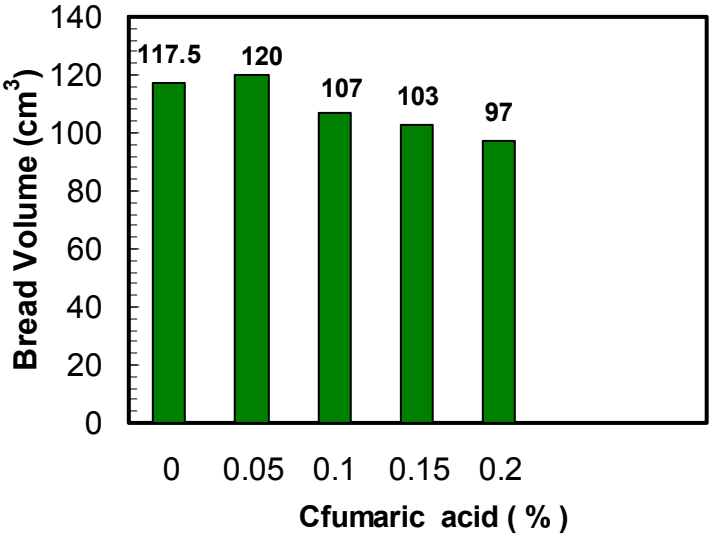

Figure 5-11 The effect of fumaric acid on bread volume

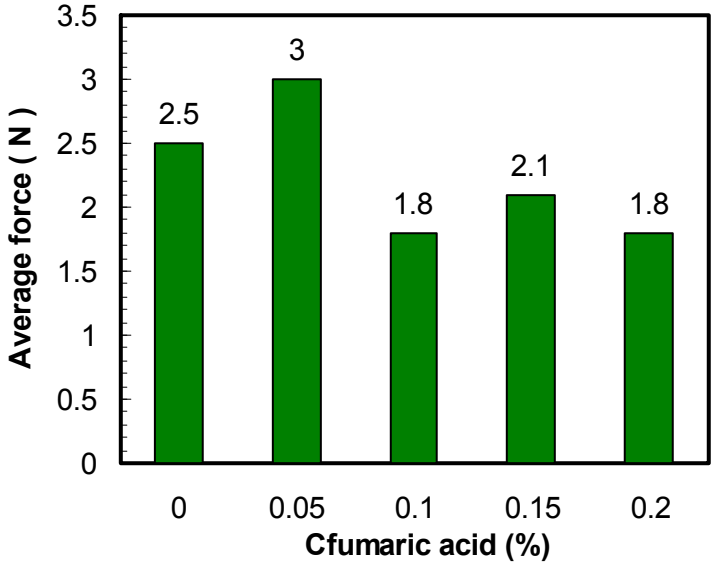

Figure 5-12 Effect of fumaric acid on bread texture

5.6 Effect of α-Amylase

Among the carbohydrases in cereals are α-amylases, β-amylases, debranching enzymes, cellulases, β-glucanases, and many glucosidases. α-Amylase appears to be the most important of these carbohydrases as it is widely used in dough formulations. [261] The enzymes most frequently incorporated during bread making are the α-amylases which

may be from different origins (cereal, fungal and bacterial). [262] It is also well established that β-amylase is prevalent in wheat flour made from unsprouted grains, however the amount of α-amylase is negligible and it is therefore necessary to supplement these flours with a certain amount of α-amylase. [263] In this study, the influence of added α-amylase has been established by application of the baking test.

5.6.1 Effect of α-Amylase on bread volume

The addition of α-amylase increases bread volume (Figure 5-13). The increase is due to the action of α-amylase, because during mixing, relaxation, scaling, dividing and proofing, α-amylase act on damaged starch producing fermentable sugars: these can be utilised by the yeast to multiply and produce carbon dioxide, necessary to inflate the dough and increase bread volume. [264] During dough mixing damaged starch swells and becomes more readily soluble. This, in addition to possible increase in starch protein interaction, results in increased dough strength; this in turn makes it more difficult for the dough expand as the gases are produced during fermentation. [265] The addition of α-amylase results in hydrolysis of damage starch reducing the strength of the dough allowing the gases to inflate the dough and increase bread volume.

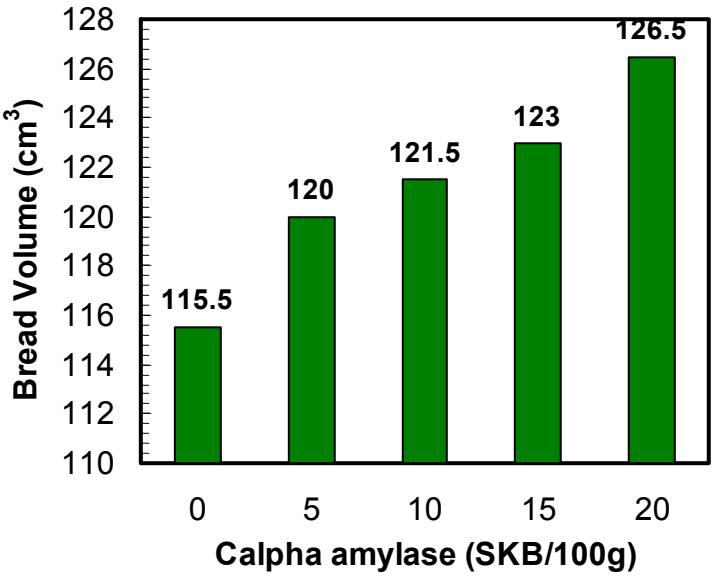

Figure 5-13 The effect of α-amylase on bread volume

5.6.2 Effect of α-amylase on bread crust colour

The addition of α-amylase affects the darkness of the crust. (Figure 5-14) Three different measures were used (L value, L-b and colour index) and in each case decreases were observed indicating an increase in crust darkness. α-Amylase digestion of damaged and gelatinsed starch produces maltose, and various sizes linear and branched dextrins [266] and these reducing sugars are then able to participate in Maillard reactions to produce the brown crust color. Appropriate levels of amylolytic enzymes are especially important during bread making for the formation of dextrins for this purpose. [267] Maillard reactions are nonenzymatic reactions involving the reaction of reducing sugars with amino groups during baking and a variety of other food processes. [268]

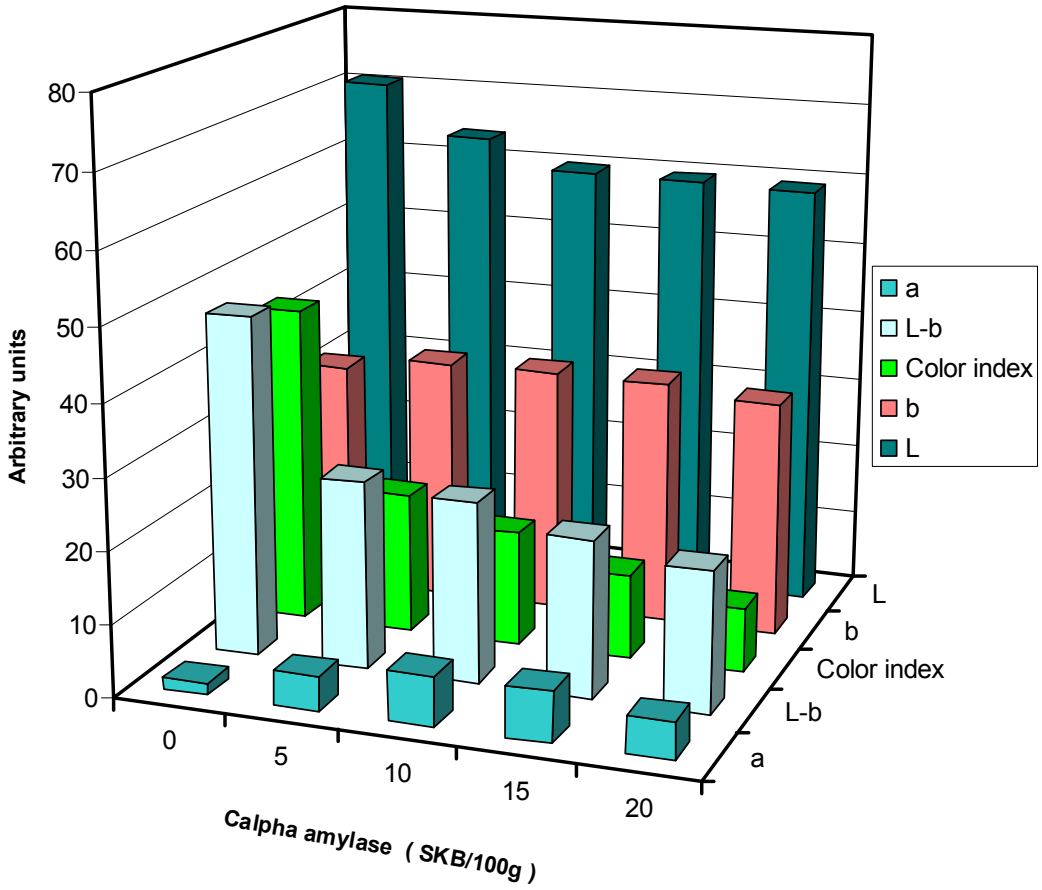

Figure 5-14 The effect of α-amylase on bread crust color

5.6.3 The influence of α-amylase on bread staling

Staling is the general term that describes the textural and flavour changes that occur in bread from the time that the loaf is removed from the oven and cooling commences. [269]

During storage, changes in flavour and, normally, a loss of the freshly baked aroma take place. [270] In our study, as illustrated in Figure 5-15, the addition of α-amylase decreases bread firmness. During storage of the trial loaves, the samples into which α-amylase had been incorporated was slower than those samples where no α-amylase was used. In addition, the rate of staling was found to be dependant on the level of incorporation.

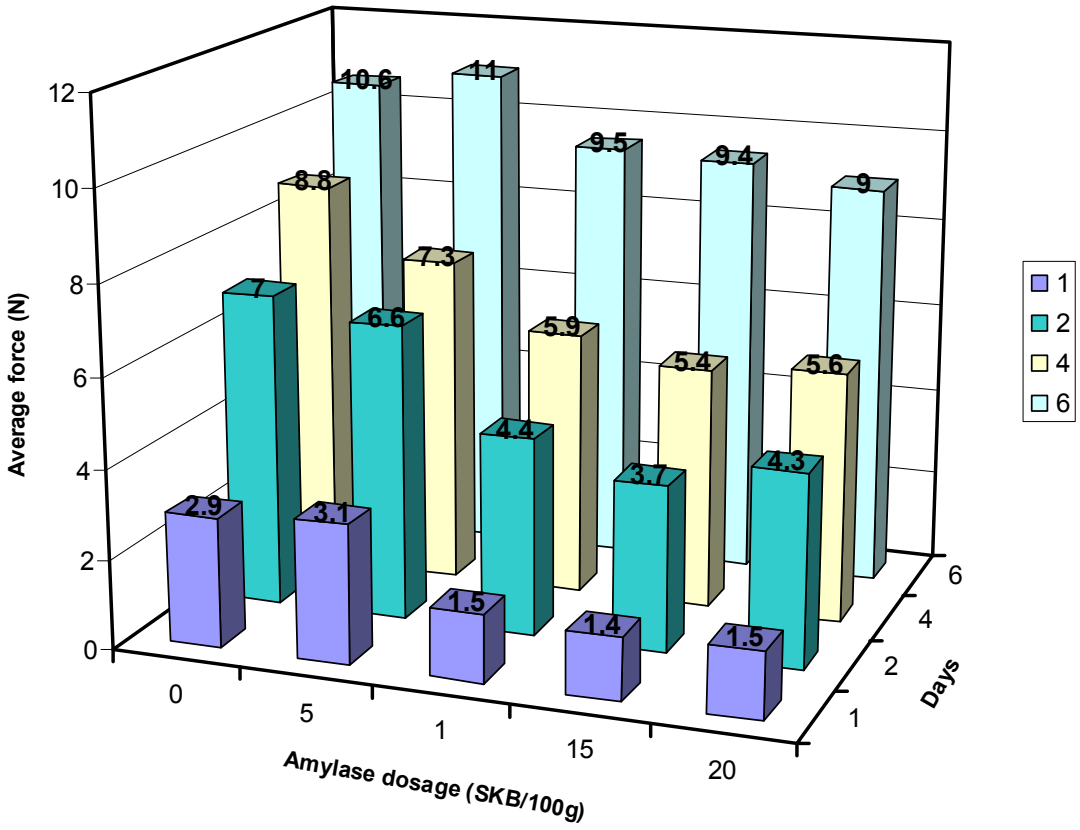

Figure 5-15 α-Amylase and it influence on bread staling

6

6 Results and discussion: the interactions of α-amylase during the fermentation process

6.1 Summary

Wheat flour doughs are complex systems in which the various molecular interactions influence the ultimate appeal of the resulting baked products. In the current study, vibration spectroscopy based upon ATR/FT-IR in the region of 800- 1300 cm^{-1} (C-C, C-O) has been used to study the interactions of α-amylase with starch in a dough system. The kinetics of the breakage of the glycosidic link and starch damage content as well as mono and polysaccharides produced during the reaction were monitored. The ATR/FT-IR spectrums were deconvoluted and the relative area of the individual peaks was calculated. Peaks at 1060 cm^{-1} and 1127 cm^{-1} were assigned to the C1-OH in glucose and the glucosidic link in the starch respectively. These peaks were monitored in order to follow the cleavage of the glycosidic link in starch and the subsequent formation of C1-OH. During the reaction, the relative area of the peak at 1060 cm^{-1} increased from 0% in the blank dough to 10% after 25 min reaction time, while the peak at 1127 cm^{-1} decreased from 2.4% to 1.7%. The peak at 976 cm^{-1} assigned to the axial deformation of C-OH in starch. This peak decreased from 15% to 5% during the same time span. These results provide a novel means of monitoring the baking process.

6.2 Introduction

Wheat is a versatile crop with the unique property that the dough prepared using its flour has the ability to be processed into a range of foodstuffs notably bread, other baked products and pastas. [110] Starch and protein are the two major constituents of wheat flour. In the case of bread, the significance of the starch will show up during each of the

major processing stages: milling, dough mixing, fermentation as well as the oven baking step and finally the storage of bread. [271]

6.2.1 Starch composition

In starch the two major constituents are amylose and amylopectin. Amylose is a linear polymer consisting of up to 6000 glucose units with α, 1-4 glycosidic bonds. Amylopectin consists of short α 1-4 linked linear chains of 10–60 glucose units and α, 1-6 linked side chains with 15–45 glucose units. [272] The kinetics and the products of the breakage of these gulcosidic bonds by exo and endoamylases have significance in starch processing in a variety of different industries.

6.2.2 Amylases mode of action on starch

Endoamylases cleave α-1,4 glycosidic bonds in amylose and amylopectin chains in a random fashion and α-amylase is well-known as having the mode of action of an endoamylase. Exoamylases, including β-amylase, amyloglucosidase, glucoamylase and α glucosidase cleave either of both of the glycosidic bonds in starch, that is the α-1, 4 and α-1, 6. [198] These glucosidic bonds in starches within the intact starch granule are stabilised by the formation of intra and intermolecular hydrogen bonds rendering starch insoluble in cold water and often resistant to both chemical and enzymatic treatments. However, starches can be gelatinsed by heating in water, which increases their chemical reactivity to the action of amylolytic enzymes. [273] This is because heat weakens the intermolecular hydrogen bonds. Unlike native starch granules, which are semicrystalline, insoluble and as a consequence inaccessible to α-amylase, damaged starch is amorphous, soluble [274] and very susceptible to attack by α-amylase enzymes. Thus damaged starch effectively provides a further supply of sugars to the yeast during the fermentation process if an active α-amylase is also present.[275]

6.2.3 Features of ATR/FT-IR

In this study the breakage of the glucosidic linkage and the production of various sugars during the reaction were monitored in a dough system using ATR/FT-IR. The theory of ATR/FT-IR has been reviewed earlier and is also available elsewhere. [164, 276] Some of the general features of ATR/FT-IR in relation to this study are that it is a type of internal reflection spectroscopy in which the sample is placed in contact with an Internal Reflection Element (IRE) of high refractive index. IR radiation is focussed onto the edge of the IRE, reflected through the IRE, and then directed to a suitable detector. [164] The depth of

penetration depends upon the wavelength of incident radiation (λ1), the angle of incidence (θ) and the ratio of the ATR crystal and sample refractive indices (n2/n1). The equation relating these parameters is presented in Figure 6-1. [277, 278] The depth of penetration of radiation is relatively shallow for many types of samples and for dough, it has been calculated to be in the order of 0.5–4 μm. [279]

$$d_p = \frac{\lambda}{2\pi n_1(\sin^2\theta - (n_2/n_1)^2)^{1/2}}$$

Figure 6-1 The equation for the depth of penetration of the ATR/FT-IR radiation. [278]

Several researchers have previously reported different applications for ATR/FT-IR in starch studies [199, 218, 280-286]. In the current study ATR/FT-IR was used to monitor the hydrolysis of starch by α-amylase in a dough system using a water absorption chosen as similar to that applied in the baking of yeast leavened bread.

6.3 Wheat flour dough spectra

Figure 6-2 shows the main peaks in the dough spectra and these have been assigned to the main components of wheat flour dough, namely water, starch, protein, and fat. The peak at 3300 cm^{-1} originated from the stretching of OH and N-H bonds. [187] While the peak at 1743 cm^{-1} is due to the carbonyl stretching of the fatty ester groups from lipids. [279] Water also absorbed strongly at around 1645 cm^{-1} due to OH bending, which is also within the amide 1 region (1600-1700 cm^{-1}). The bands in this particular region of the spectrum are very sensitive to conformation changes in protein secondary structure. The results presented in chapter 6 showed valuable information about the intermolecular interaction of baking ingredients and their impact on wheat flour protein secondary structure. Amide 1 originated from C=O stretch and N-H vibration modes. [195] To eliminate the interference of water with Amide 1 the band at 2125 due to OH deformation has been used for water subtraction procedure effectively representing an internal standard.[221] The region from 1500-1600 is Amide 11 which is primarily N-H bending, C-N stretching modes, and it is particularly sensitive to protein- solvent interaction and protein–protein hydrophobic interaction. [287] The region from 1300-800 cm^{-1} (C-C, C-O) corresponds to the starch along with its hydrolysis products, and this region is not perturbed by water. [200] This is a result of the water absorption band due to OH deformation appearing in the region below 800 cm^{-1} [279] so that no water subtraction procedures were needed.

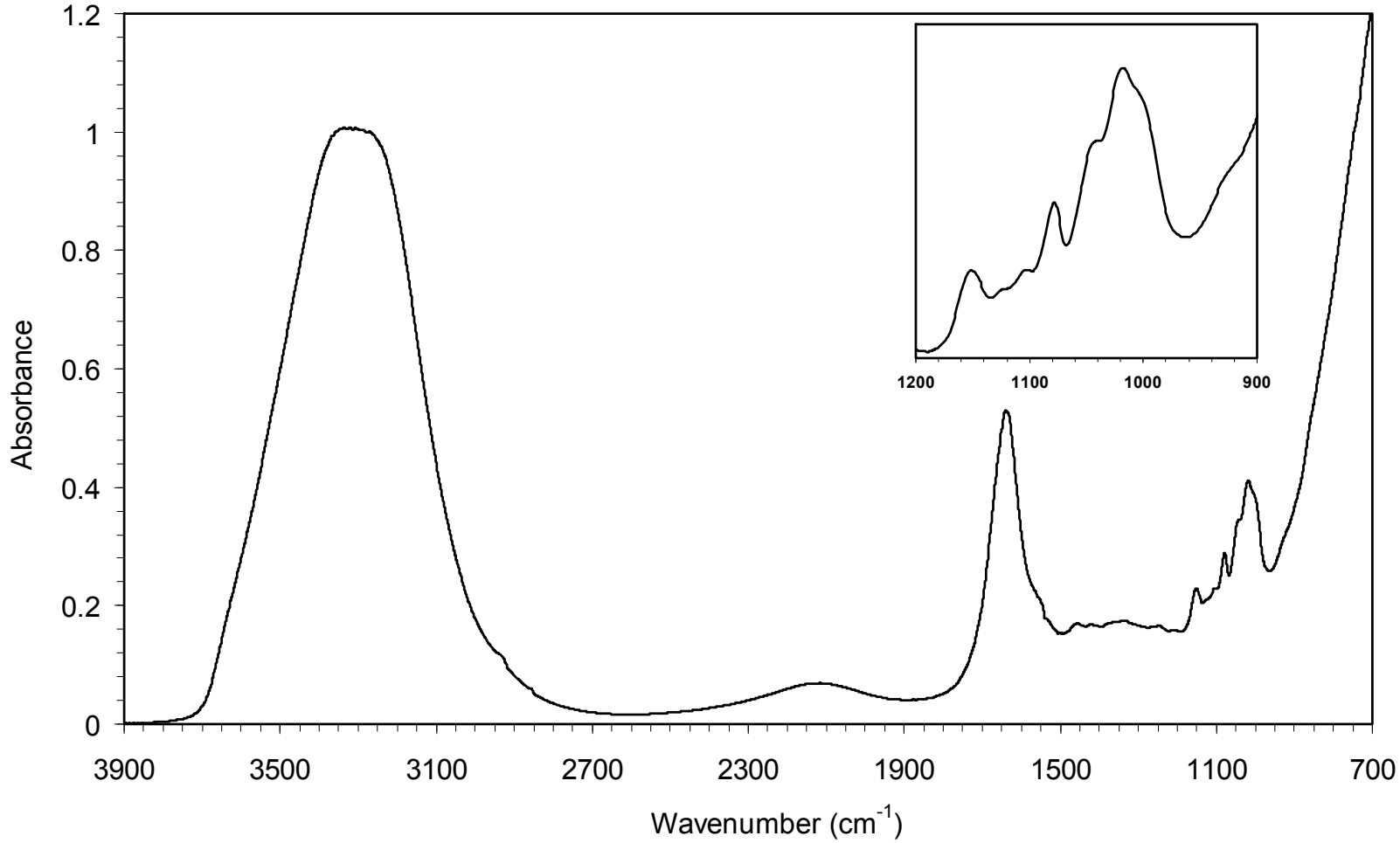

Figure 6-2 A typical wheat flour dough ATR/FT-IR spectrum

6.4 α Amylase hydrolysis product spectrum

The deconvoluted spectrum of the blank dough (Figure 6-3) is presented in Figure 6-3. The main peak observed in the spectra for the product involving α-amylase hydrolysis (Figure 6-4 to Figure 6-9) and not observed in the blank dough spectrum (Figure 6-3) is the peak at 1060 cm^{-1}. This peak also appear in the spectrum obtained for pure glucose. As one of the main peaks featuring in the D-glucose spectrum, this peak is assigned to the bending of C1-OH in the D-glucose molecule. [200-202] Careful inspection of Figure 6-4 to Figure 6-9 indicates that the area under this peak increases as the incubation time is extended.

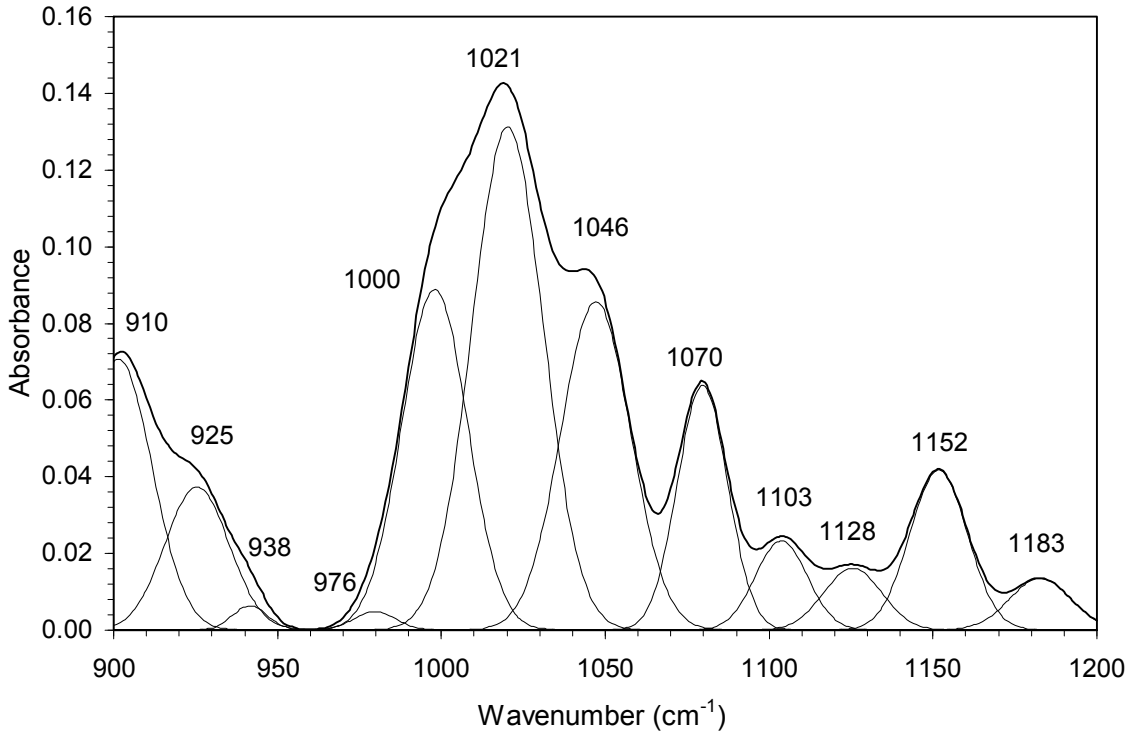

Figure 6-3 The deconvoluted blank dough spectrum before the addition of α-amylase.

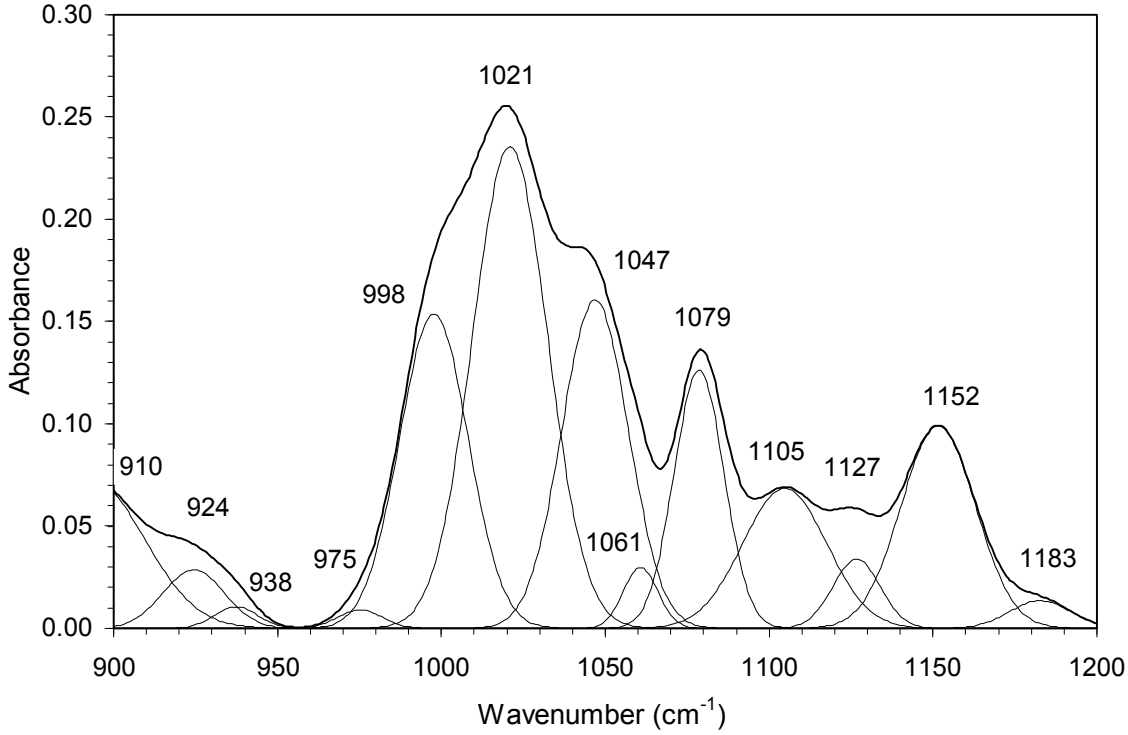

Figure 6-4 The deconvoluted dough spectrum immediately after the addition of α-amylase.

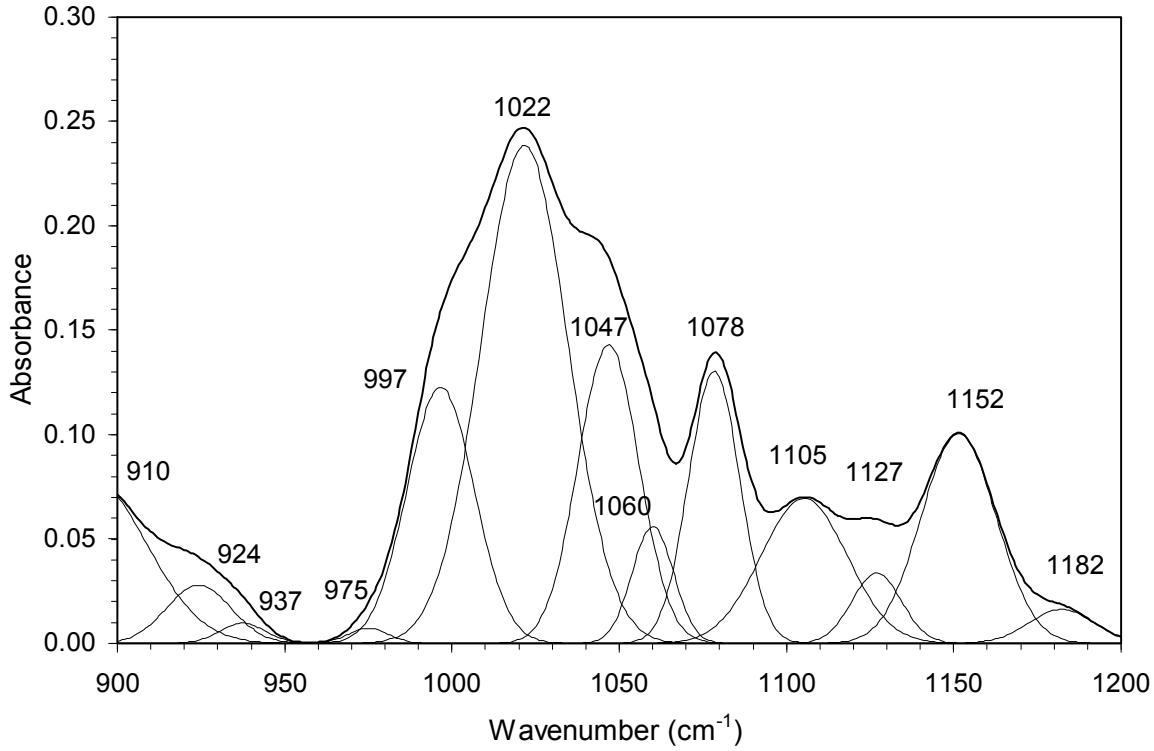

Figure 6-5 The deconvoluted dough spectrum after 5 min of the α-amylase reaction with dough.

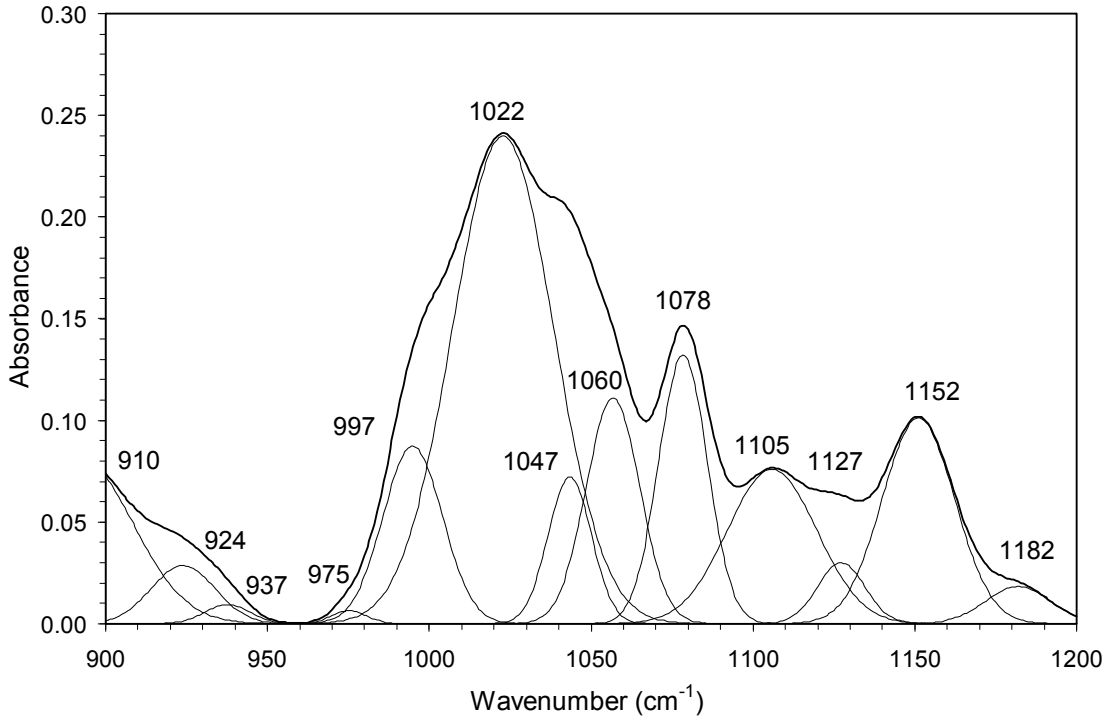

Figure 6-6 The deconvoluted dough spectrum after 10 min from initiation of the α-amylase reaction with dough.

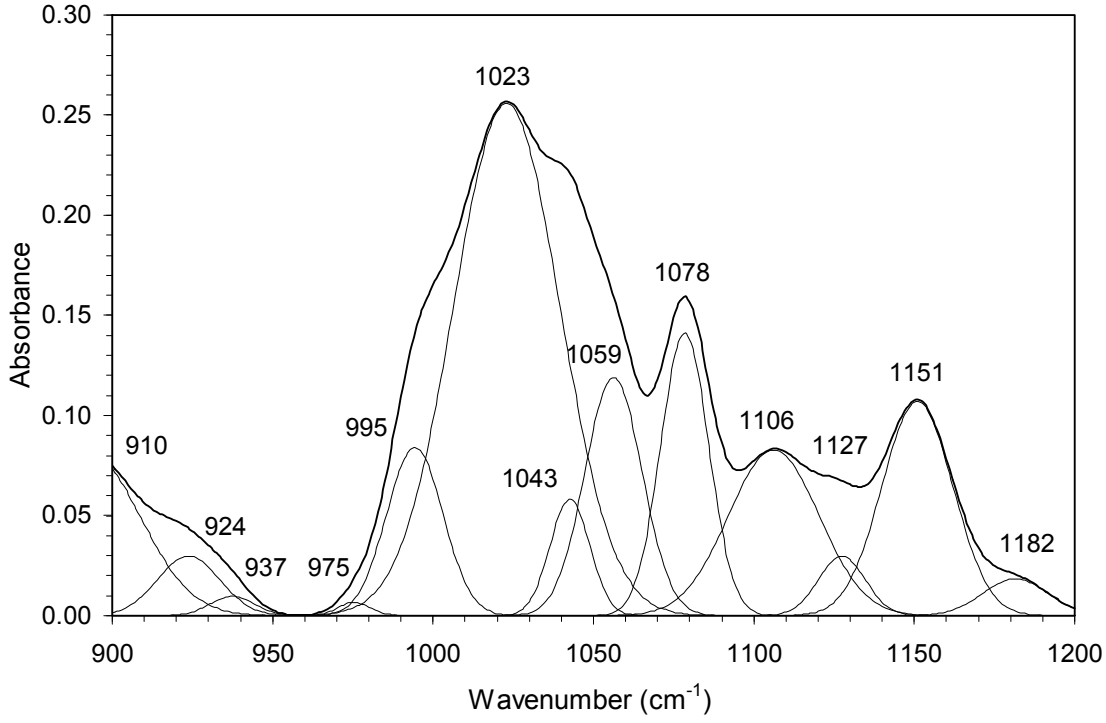

Figure 6-7 The deconvoluted dough spectrum after 15 min of the α-amylase reaction with dough.

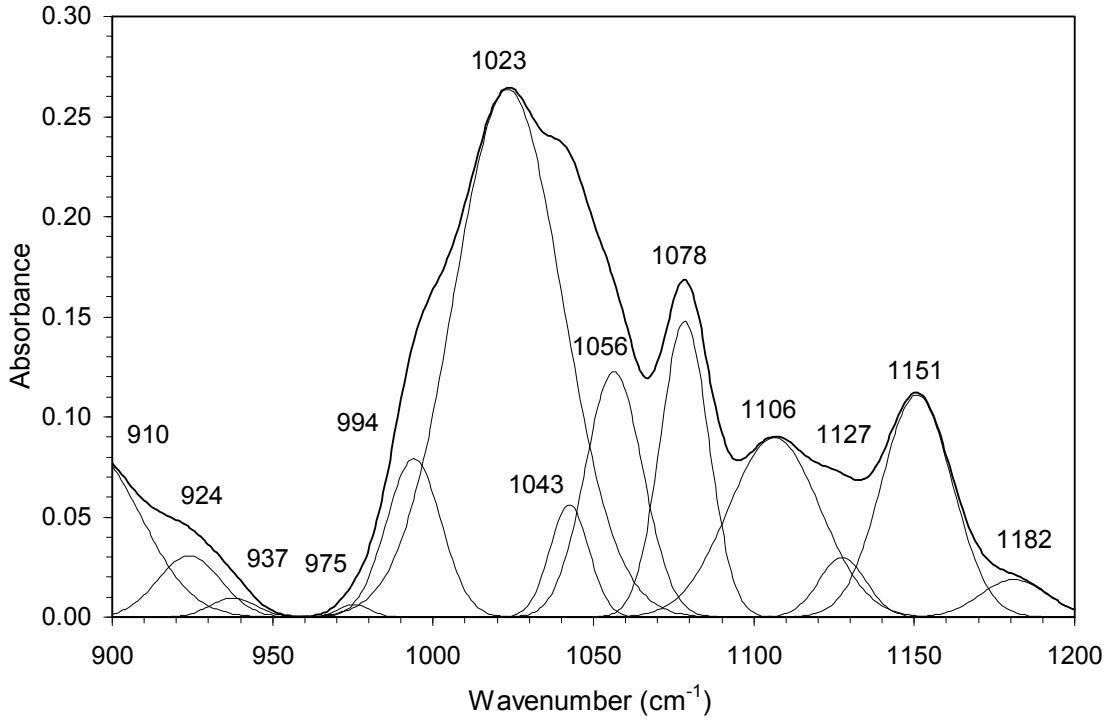

Figure 6-8 The deconvoluted dough spectrum after 20 min of the α-amylase reaction with dough.

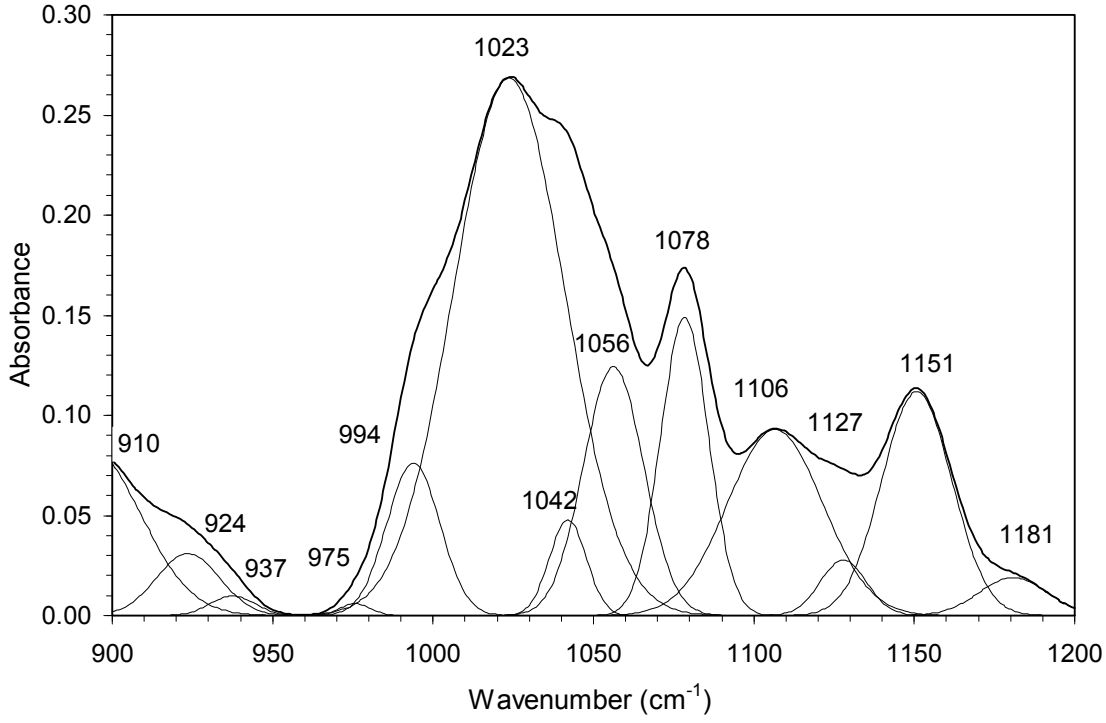

Figure 6-9 The deconvoluted dough spectrum after 25 min of the α-amylase reaction with dough.

6.5 Monitoring the breakage of the glycosidic linkage (α-1, 4)

Interesting changes in relation to the action of α-amylase on starch is seen through the peak at 937 cm^{-1} which is specifically assigned to the C-O-C in α-1, 4- linkage in the glycosidic chain of starch.[200, 202, 204, 211, 288] The relative area of this peak decreased from 0.67 % at the initiation of the reaction to 0.54 % after 25 minutes of reaction time. The kinetics of the breakage of the glycosidic linkage in starch is presented in Figure 6-10, showing that there is a linear relation between time and the breakage of the glycosidic linkage. The shape of the graph is very similar to that observed for glucose production (Figure 6-16), the difference being that the glucose is seen to increase and the glycosidic bond decreases coinciding with the mode of action of α-amylase in producing glucose as a product of the breakage of the glycosidic linkage in starch polymer.

One of the purposes of α amylase addition in wheat flour dough is to break the starch molecules, producing glucose so this can permeate through the yeast cell wall and be utilised as a carbon source for the yeast. This results, in turn in the production of carbon dioxide as a by-product which has the important function of inflating the dough during the fermentation process. So in the light of these results, we have been able to monitor the fermentation process with the changes from the addition of water to the flour, up to the proofing stage, Including the kinetics of the breakage of the glycosidic linkage in wheat flour starch which will be of great interest to cereal chemists in order to monitor the effect of various ingredients on the kinetics of the breakage of the glycosidic linkage, more over the level of α amylase can effectively be quantified.

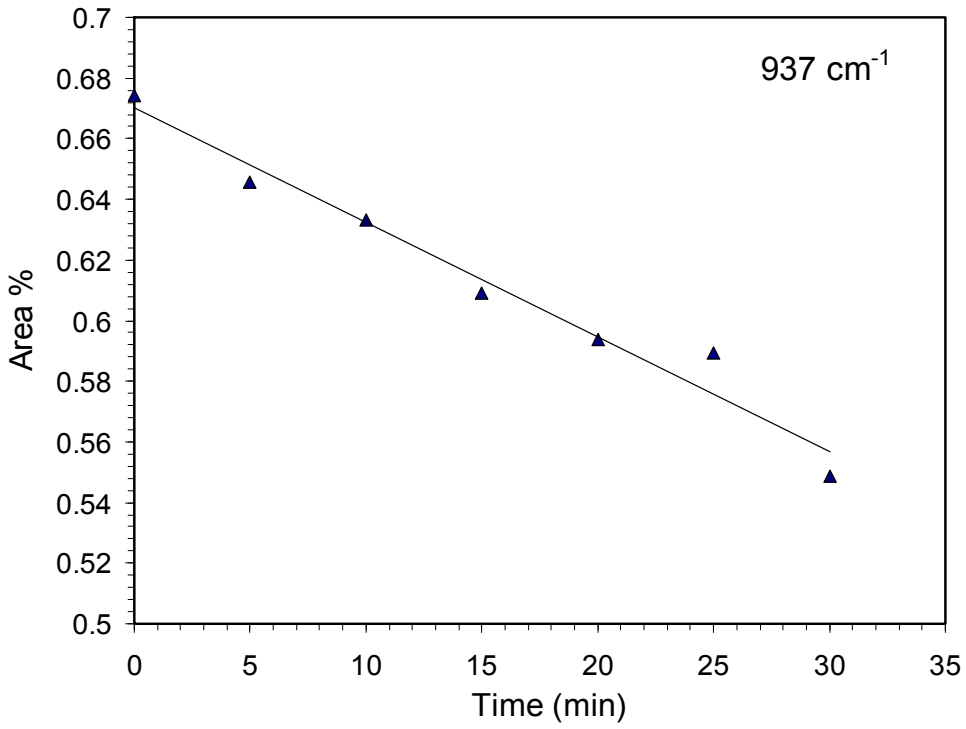

Figure 6-10 The kinetics of the α-amylase reaction in dough based on the 937 cm^{-1} (glycosidic linkage α-1,4) relative peak area obtained from the deconvoluted spectra

6.6 The kinetics of the α-amylase reaction with dough based on the peak at around 976 cm-1

The relative area of the peak at 976 cm^{-1} decreased from 0.43% at the beginning of the reaction to 0.29% after 25 min of reaction time, Figure 6-11 shows the plot of the relative area of this peak versus reaction time. Similar results for the time dependent acid hydrolysis of some oligosaccharides were reported by Mathlouthi. [200] The band most affected in the previous study was situated in the 993-966 cm^{-1} range and corresponded to the stretching of the C-O bond within the C-O-C structure. The vibration in this region can be assigned to the glycosidic linkage, and could also be assigned to the deformation of the axial C-OH at C-4 (C-4-OH). [200] We stongly suggest that there is a correlation between this peak and the level of starch damage in wheat flour, because under the reaction conditions used in the current study, the most susceptible of the glycosidic linkages likely to be present in the dough system, is the glycosidic linkage in the damage starch components because this is amorphous, soluble and highly susceptible to α-amylase action while intact starch granules are semi crystal, insoluble and inaccessible to α-amylase. [274] Other changes in the dough spectra include those in the peak at around 997 cm^{-1}.This peak is water sensitive and the changes of this peak are presented in Figure 6-12.

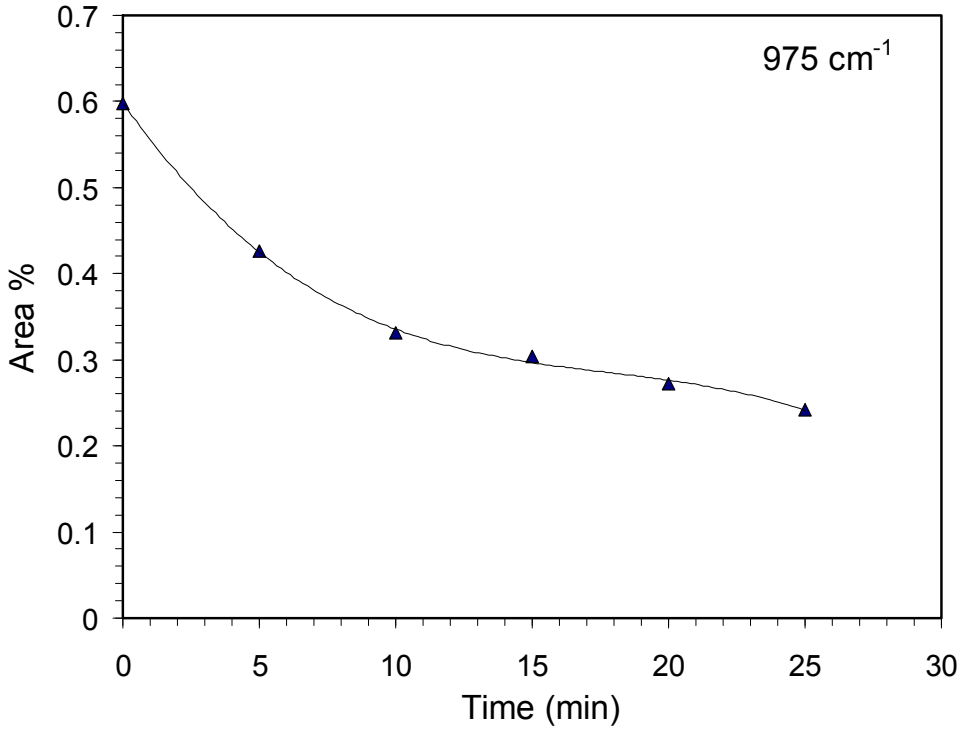

Figure 6-11 The kinetics of the α-amylase reaction with dough based on the 975 cm^{-1} relative peak area obtained from the deconvoluted spectra

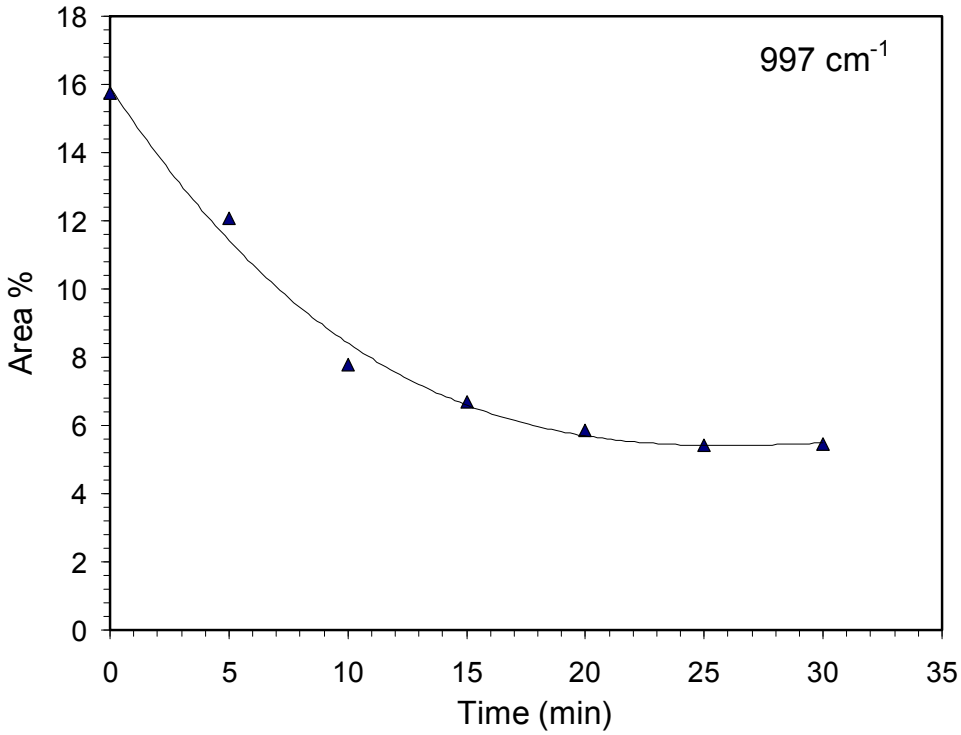

Figure 6-12 The kinetics of the α-amylase reaction with dough based on the relative area of the 997 cm^{-1} peak obtained from deconvoluted spectra

6.7 Effect of the enzymatic degradation on starch amorphous and crystalline structure

Changes in starch structure during the reaction course were also investigated by monitoring the changes in the peaks at 1022 cm^{-1} which is characteristic of amorphous starch and the peak centred at 1043 cm^{-1} characteristic of the more organized part of starch (crystal structure). [218] The peak at 1022 cm^{-1} (Figure 6-13) increased from 26.4% at 0.0 min to 39.9% after 25 min indicating that the relative amounts were greater for the more amorphous structure and these were generated in starch during the reaction. Corresponding to this, the relative area of the peak at 1043 cm^{-1} (Figure 6-14) decreased from 16% to 2.4 % during the same time span, confirming that the crystal structure was further altered during the course of the reaction. X- Ray diffractometry has commonly been used for the determination of starch crystallinity. [213] Here we used the ratio of the relative area of the peak at 1043 cm^{-1} to that for the peak at 1024 cm^{-1}. This is presented in Figure 6-15 and shows its use as a measure of wheat starch crystallinity according to the method described by Van Soest. [215] The same two peaks were used by various workers particularly including Oneh [289] who monitored the effect of irradiation on the structure of cowpea starch granules. In addition, Van Soest used a similar approach in assessing short- range structural changes in potato starch [215] and Sevenou [206] applied this to an investigation of the organisation of the external region of the starch granule.

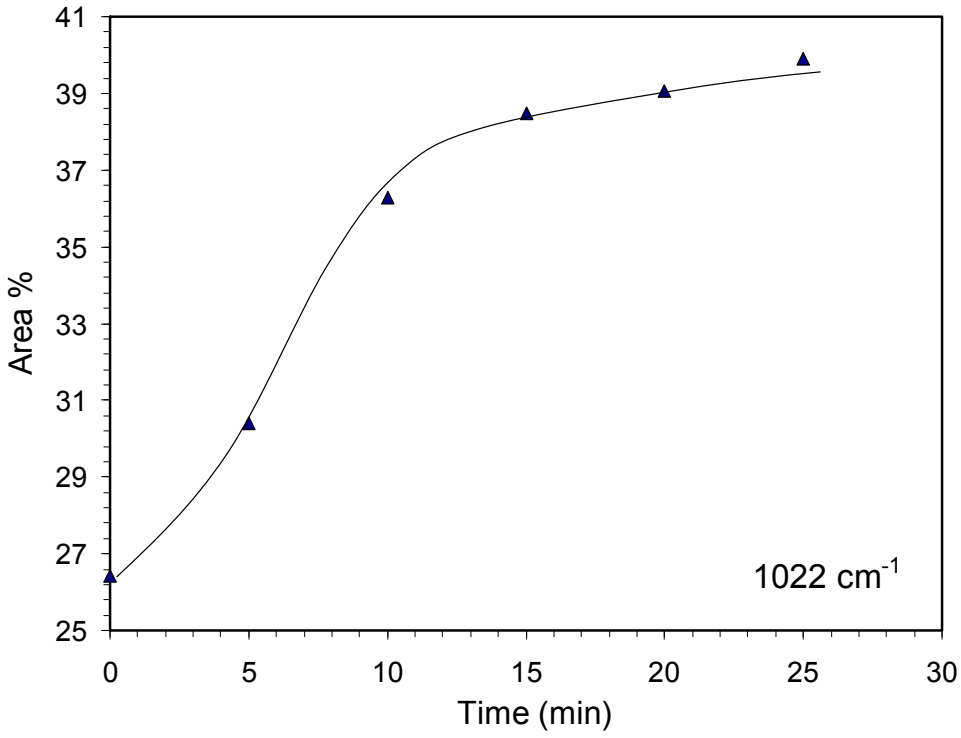

Figure 6-13 The kinetics of the α-amylase reaction with dough based on the 1022 cm^{-1} (amorphous structure) relative peak area obtained from deconvoluted spectra

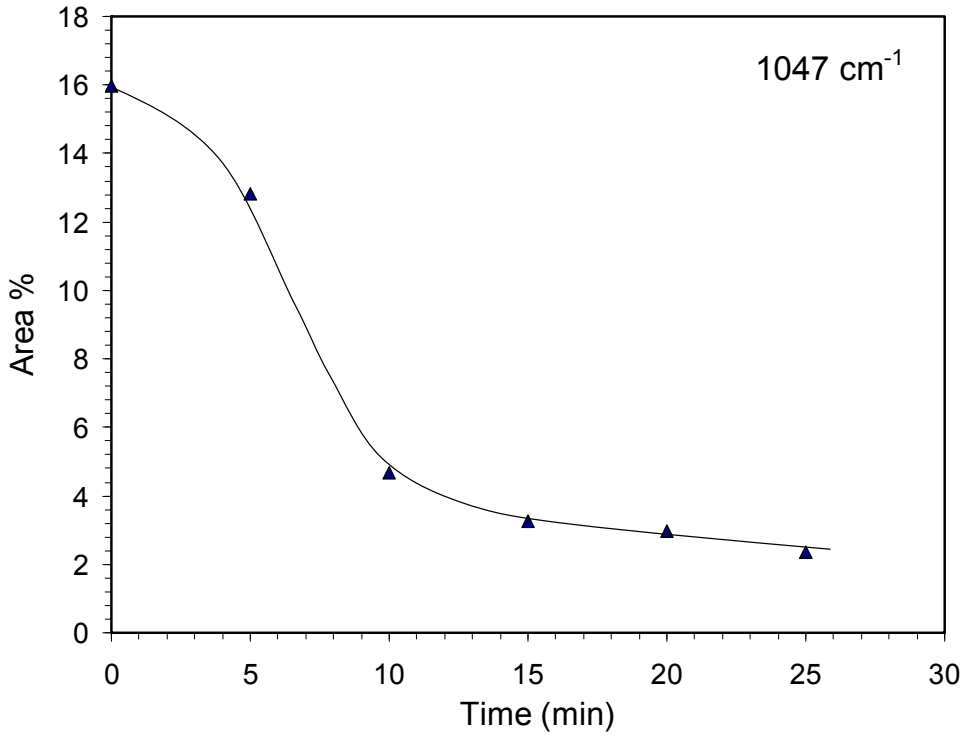

Figure 6-14 The kinetics of the α-amylase reaction with dough based on the 1047 cm^{-1} (crystal structure) relative peak area obtained from the deconvoluted spectra

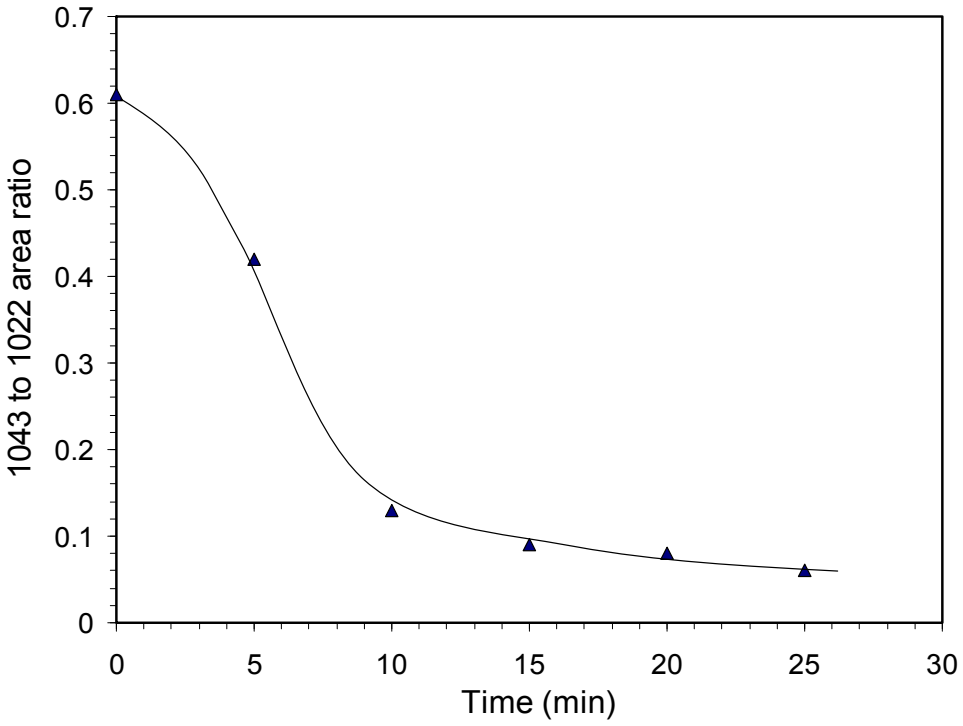

Figure 6-15 The ratio of the 1047 cm^{-1} (crystal structure) relative peak area to 1022 cm^{-1} (amorphous structure) obtained from the deconvoluted spectra.

Figure 6-16 shows the plot the relative area of the peak versus time at 1060 cm^{-1} the area of this peak increased from 0.0 to 10.01% after 25 minutes of α-amylase reaction on starch. Glucose is one of the products of this reaction and the increase in the % area of this peak can be interpreted as more free hydroxyl groups were produced during the reaction. This reflects the fact that free glucose contains five free OH groups while in the starch polymer, a monomeric unit contains, on average, fewer than three OH groups. [201] Monitoring this peak is a useful approach in that it facilitates the study of the kinetics of the action of α-amylase on starch; quantitative measurement of the total of all sugars is possible.

These results are in good agreement with the endo mode of action of α-amylase in which it cleaves α-1,4-glycosidic links at random, yielding shorter chain fragments including oligosaccharides. [201] These, in turn, can then be converted to glucose by amylases because they are soluble thereby increasing accessibility by the amylases and resulting in conversion to glucose. [290]

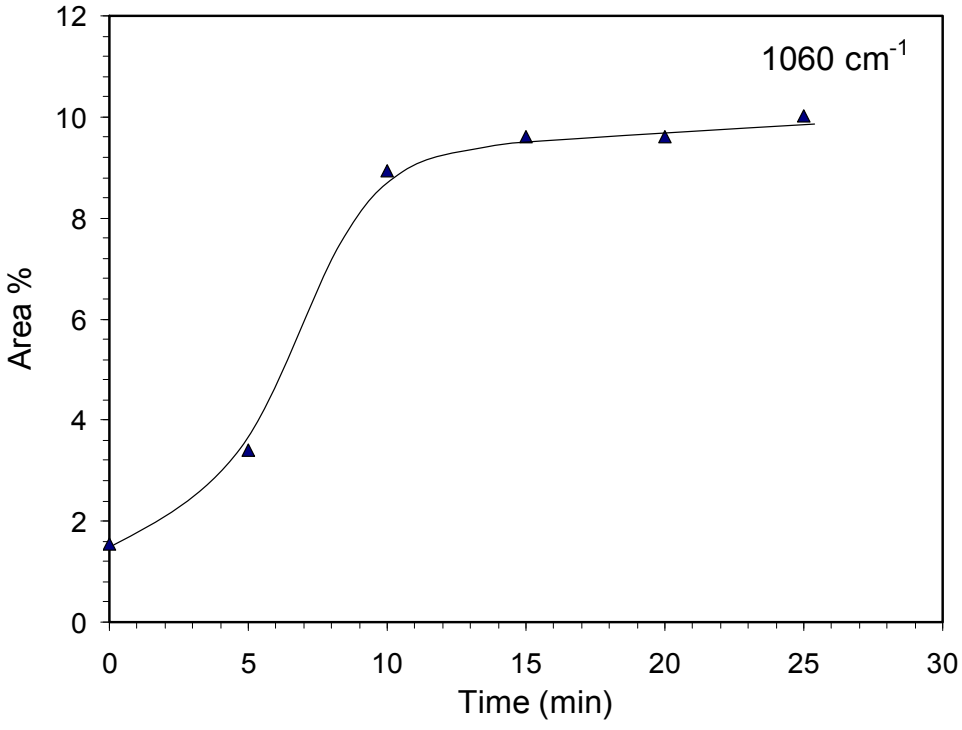

Figure 6-16 The kinetics of the α-amylase reaction with dough based on the 1060 cm^{-1} relative peak area obtained from the deconvoluted spectra

Another peak change observed to be due to the hydrolytic action of α-amylase is the peak at around 1100 cm^{-1} (Figure 6-17). This peak is assigned to the stretching of (C-O), associated

with the ring originated from C-4-O and C-6-O in glucose. [200, 202, 291] The relative area of this peak increased from 8.6% at the beginning of the reaction to 11.8% after 25 minutes, the increase in the % area of this peak is further evidence for the increase in the amount of free glucose in the reaction medium. Figure 6-17 shows that there is a linear relation between the % area of the peak at 1100 cm^{-1} and the reaction time. Chromatographic analysis of the hydrolysis products has shown that glucose is the main product of the reaction between starch and α-amylase. [273] Other changes in the dough spectra are seen in the peak at 1127 cm^{-1} and presented in Figure 6-18.

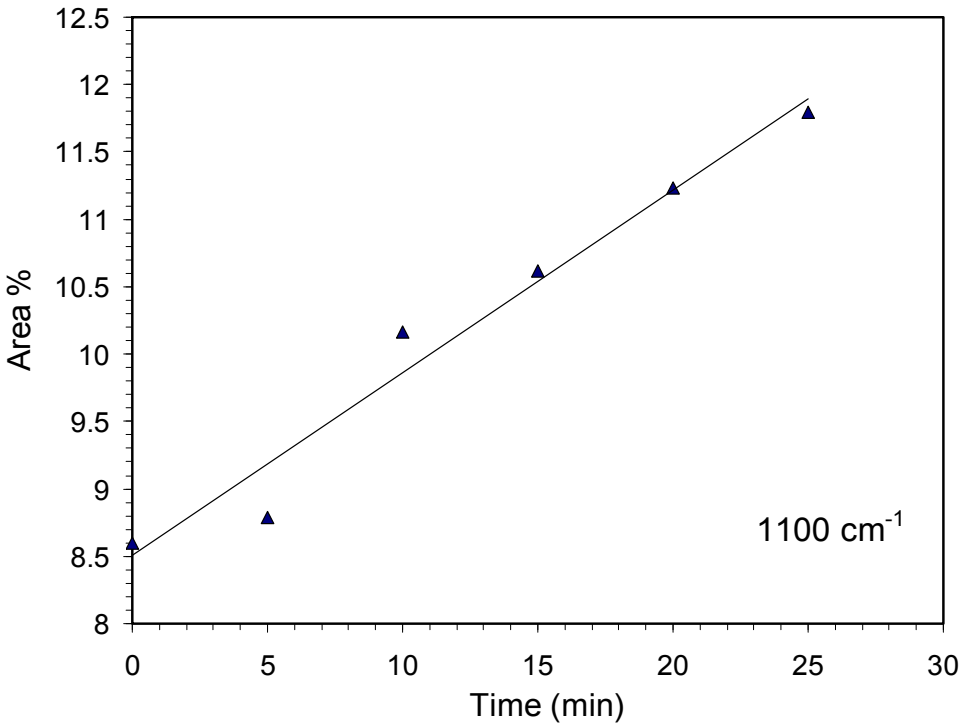

Figure 6-17 The kinetics of the α-amylase reaction with dough based on the 1105 cm^{-1} relative peak area obtained from the deconvoluted spectra.

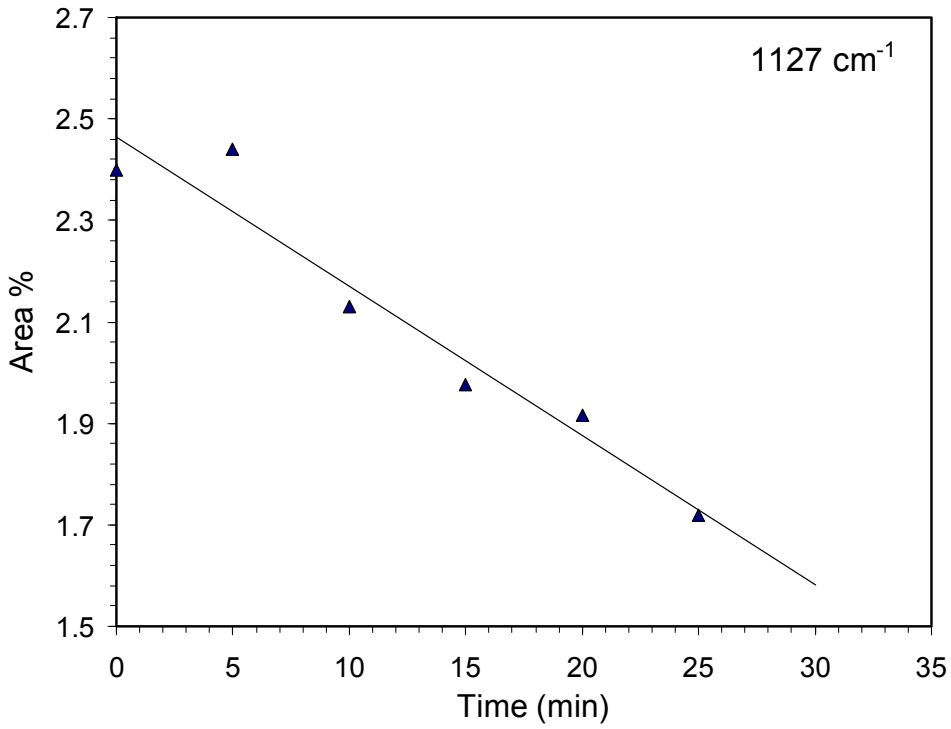

Figure 6-18 The kinetics of the α-amylase reaction with dough based on the 1127 cm^{-1} relative peak area obtained from the deconvoluted spectra.

Another band affected by the hydrolytic action of α amylase is the peak at 1151 (Figure 6-19). The vibration in this region originates from the coupling of the stretching of (C-O), the bending of (C-H) and the stretching of (C-O-H). [200] The area of this peak decreased from 11.0 to 10.4% after 25 min of reaction time corresponding to the decrease in the intensity of this peak as a consequent of the hydrolytic action of α amylase on starch that has been reported by Krieg. [202, 292] In attempting to determine the activity of α amylase in human serum samples, by recording two spectra, one immediately after the addition of the enzyme to starch, and one after 20 min reaction time and taking the difference in the spectra, this peak was one of the main peaks giving rise to negative peaks in the their study of the difference spectra.

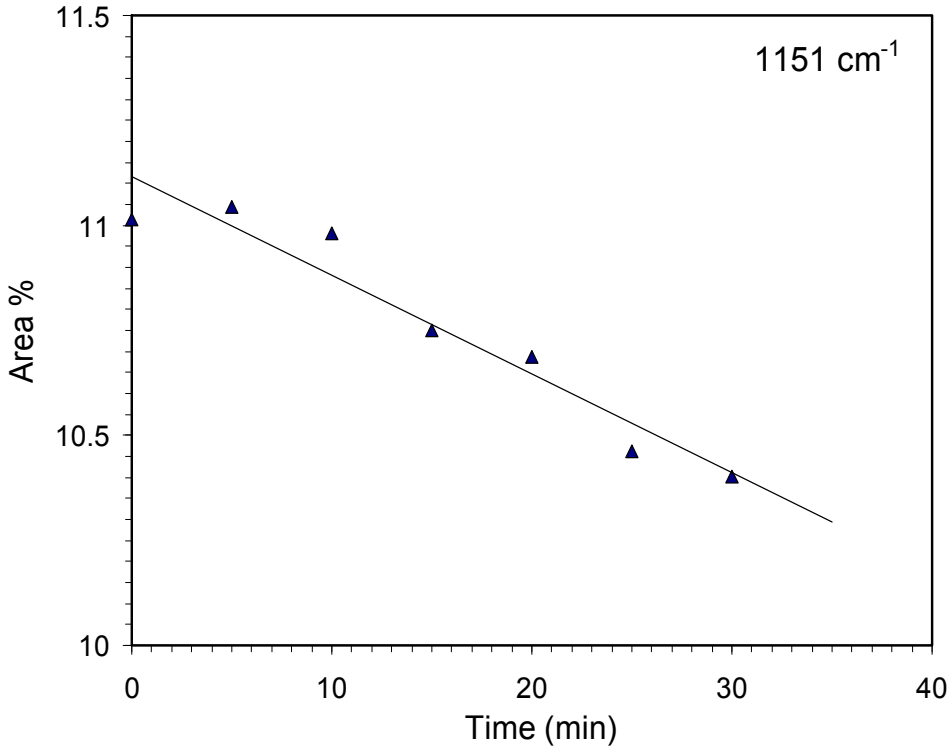

Figure 6-19 The kinetics of the α-amylase reaction with dough based on the 1151 cm^{-1} relative peak area obtained from the deconvoluted spectra.

6.8 Conclusion

ATR/FT-IR is a useful technique in studies of starch hydrolysis and quantitative measurement of sugar in the dough system. The various ingredients and conditions affecting the kinetics of the breakage of the glycosidic linkage were monitored by using ATR/FT-IR. The effect of dough ingredients on starch can be monitored and measured. The structural changes and intra and inter molecular interactions accompanying the reaction catalysed by the α amylase greatly affect the appealing and characteristics of the food products made from wheat flour doughs. The whole fermentation process can effectively be monitored using ATR FT-IR.

7

7 Results and discussion: the application of ATR/FT-IR to an investigation of inter- and intra-molecular interactions and protein secondary structure in a dough system

7.1 Introduction

The purpose of this study has been to investigate the effect of the widely used oxidising agent ascorbic acid and the reducing agent L-cysteine on dough structure. These are important as they influence the ability of dough to retain the gases produced as a by-product when yeast utilise the sugars produced by the action of α-amylase on starch. This chapter focuses on the amide I and amide II components and relates these with the characteristics of the dough. It also investigates the changes in inter-molecular and intra-molecular interactions upon addition of the selected oxidising or reducing agent, so it seeks to elucidate information from the whole spectrum of the wheat flour dough.

Plant proteins play significant roles in human nutrition. [293] Wheat is one of the most widely cultivated plants. [92] Gluten proteins are the predominant proteins in wheat flour; these are insoluble in water under conditions in which the pH values are near-neutral as well as having viscoelastic properties when hydrated. [294] Gluten contains two distinguishable fractions: the glutenins (disulfide -linked polymers) and the gliadins (typically monomeric proteins) which together give dough a combination of strength and extensibility. [196] Gliadin contributes particularly to the viscosity of the dough while glutenin provides elasticity characteristics to the dough. Gliadins were initially classified into four groups on the basis of mobility at low pH during gel electrophoresis (α-, β-, γ-, ω- gliadins in order of decreasing mobility). [115] Glutenin subunits, on the other hand, consist of two distinct groups, the

predominant low-molecular-weight species and the high molecular weight (HMW) subunits. [287]

Dough, in its most basic form, is made by combining water, flour, and energy. [295] Water causes the proteins to swell and mechanical energy promotes organisation of the proteins into a continuous matrix giving dough a unique viscoelastic structure. [296] Dough also can be defined as a composite system that comprises two dispersed filler phases (gas cells and starch granules) and the gluten matrix. The relative proportions of these and the rheological properties of the matrix are a reflection of those of the fillers and these all contribute in determining the behaviour of the composite. [297] Additives and technological aids (also referred to as processing aids) are extensively used in the baking industry for the enhancement of the dough handling properties and machinability. [226]

L-Cysteine and similar thiol compounds play an important role in the modification of the rheological properties of foods. [249] L-cysteine reduces the disulfide bonds between proteins and, in a dough, weakens the strength of the gluten network in the dough. [298] The usefulness of ascorbic acid on the other hand depends on its ability to modify the properties of the proteins of wheat flour in a technologically advantageous manner. [299]

Many attempts have been made to establish the biochemical basis for the baking quality of wheat flour. [300] A wide variety of methods have been used by other researchers in an attempt to quantify the effect of oxidising and reducing agents on wheat flour dough. However those methods do not give direct information about the effect on protein secondary structure. [252, 301, 302] The basis of the studies reported here is that a vibrational spectrum can give a more complete picture of the structure (geometry, electronic structure) and dynamics of an analysed molecule. [177]

IR spectroscopy was one of the earliest experimental methods recognised as potentially useful for estimating the secondary structure of polypeptides and proteins. [174] Over the past decade, more recent developments in methods for analysis of spectral data have revitalised the use of ATR/FT-IR spectroscopy in protein analysis. Several different techniques are available for the quantification of protein secondary structure from ATR/FT-IR spectra of proteins. [303] This includes methods for accurately quantify protein secondary structure [304] and there are now three analytical procedures currently in use: Fourier self deconvolution, second derivative and curve-fitting. [184]

7.2 ATR/FTIR of wheat flour dough

ATR/FT-IR spectroscopy has played a pioneering role in studying the conformation of peptides, poly-peptides and proteins. [176] Its application to analysis of protein secondary structure is based on the sensitivity of the absorption bands of the peptide group to the conformation of the surrounding polypeptide chain. [305] Figure 7-1 shows the typical spectrum obtained in the current study for a wheat flour dough that might be used in breadmaking. The amide groups of polypeptides and proteins possess nine characteristic vibration modes or group frequencies [174] and these are referred to as amide A, B, I, II, III, V, VI, VII, VIII. As seen in the spectrum, the amide A band occurs at around 3500 cm^{-1} and amide B at 3100 cm^{-1}. Between 300-1700 cm^{-1} the peaks are those of amide I to amide VII starting from the higher wave number for amide I to the lower wave number for amide VII that are characteristic of secondary trans amides. [224] Of these vibrational bands, the amide I band has been the most useful probe for determining the secondary structures of proteins. [184] This particular band is due almost entirely to the C-O stretch vibration of the peptide linkages that constitute the backbone structure of a protein.

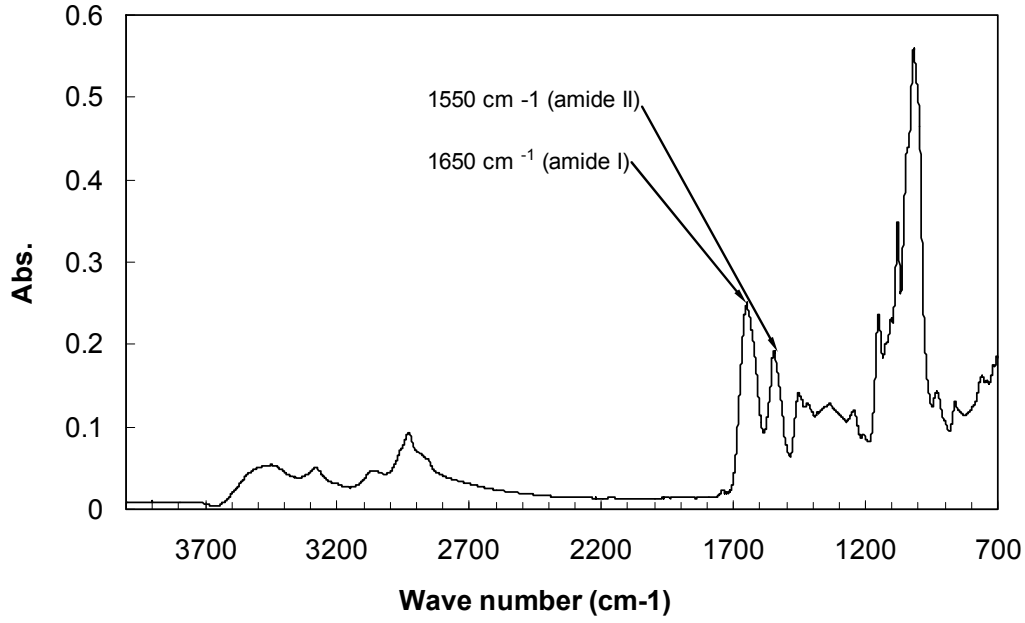

Figure 7-1 ATR/FT-IR spectrum of a typical wheat flour dough following deconvolution

7.2.1 Amide I

The regions of amide I in the wheat flour dough (1600-1700 cm^{-1}) are seen in Figure 7-1. The assignment of this peak has been based upon the report by Lee and co-workers [178] The peak for amide I is centred at around 1650 cm^{-1} Figure 7-1, although in this study it was observed that the peak did shift to a lower frequency and sometimes to higher values

depending on the specific nature of the experimental variations and the additives being used. This indicates that the food additives incorporated into the dough formulation brought about important changes in protein secondary structure. High sensitivity to small variations in molecular geometry and hydrogen bonding patterns makes the amide I band uniquely useful for the analysis of protein secondary structural composition and conformational changes. [163]

The results for the individual components of the amide I are presented in Figure 7-2. After the deconvolution and peak fitting process, the spectrum of the dough showed a prominent band at around 1650 cm^{-1} that is associated with the α-helix conformation. The band seen to occur at around 1613 cm^{-1} was assigned to the intermolecular β-sheet structure. [176, 306] In addition, the band centred at around 1630 cm^{-1} was assigned to intra-molecular β-sheet structure, [174, 188] while that at around 1670 cm^{-1} is associated with the presence of β-turns, a structure predicted in the long repetitive domains of prolamin molecules. [188] The band at 1685 cm^{-1} was assigned to anti-parallel β-sheet. [189, 195] In all of the spectra of the dough samples examined in the current study, the amide I contained deconvoluted peaks representing each of the specific conformations that have been assigned here. This has then led to the conclusion that each of the secondary structures are present within the gluten matrix in the wheat flour dough.

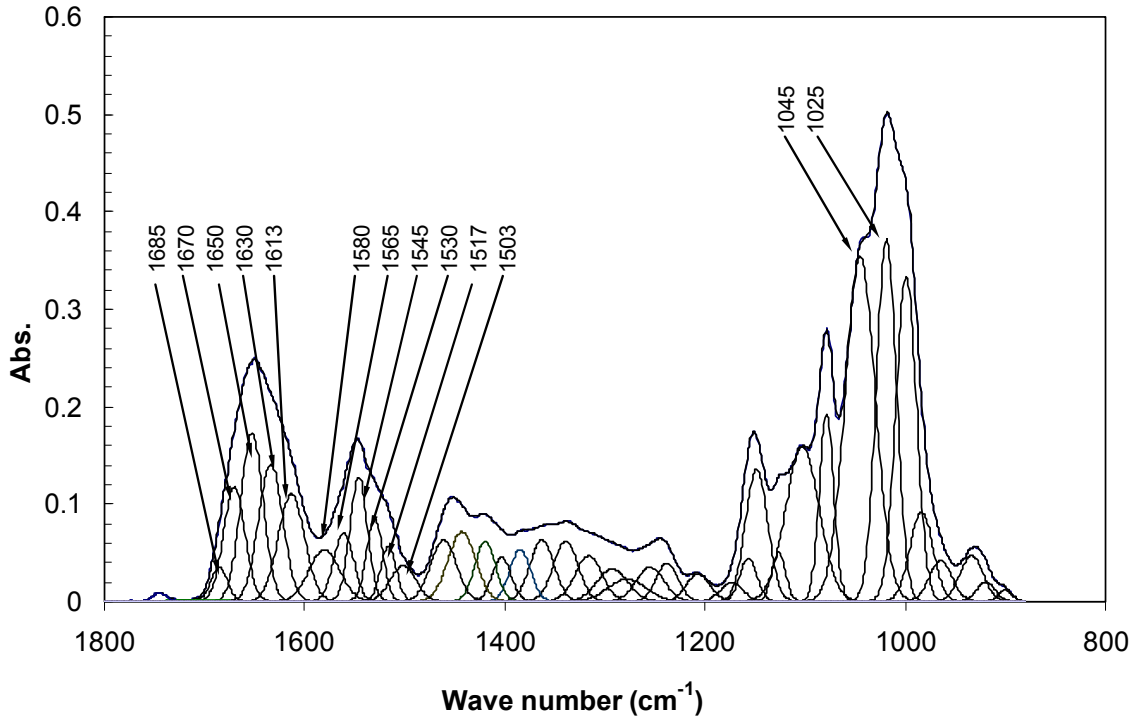

Figure 7-2 ATR/FTIR spectrum of wheat flour dough showing the amide I and II regions in the dough spectrum following deconvolution

7.2.2 Amide II

Despite the demonstrated value of the amide I region, it is noted that amide II and amide III have also been used for structural quantification, although to a lesser extent. [304] In Figure 7-2 the amide II region (1500-1600 cm^{-1}) consists primarily of N-H bending and C-N stretching modes. [287] The principal peak in the amide II band is that centred at around 1545 cm^{-1}. This peak characterising the amide II region was assigned to the secondary structure of the α-helix. [307, 308]

The other peak within the amide II which also exhibited significant changes, in this study, was that at around 1530 cm^{-1}. This peak was assigned to the β-sheet structure. [308] It was also noted that other peaks typically appeared at 1503 cm^{-1}, 1517 cm^{-1}, 1665 cm^{-1}, and 1580 cm^{-1}.

7.2.3 Water subtraction

Water molecules absorb IR at a variety of wavelengths and Figure 7-3 A shows a typical spectrum of a dough. In this is included the contributions relating directly to the water molecules present in the dough. Figure 7-3 B shows the molecular vibration modes obtained for water, and in Figure 7-3 C the dough spectrum is presented following water subtraction. In Figure 7-3 B, the absorption band seen at around 3400 cm^{-1} is due to the anti-symmetric stretching mode of vibration of water molecules. [306] In addition, the absorption band at approximately 3200 cm^{-1} is due to the symmetric vibration mode of the water molecule. [309] The band which interferes with the amide I absorption band is that at around 1640 cm^{-1} arising from the water bending vibration mode. Hence, in order to elucidate any information from the amide I band, the contribution of the absorption of this band has to be removed from the dough spectrum. Alternatively, the problem can be resolved using a deuterium oxide solution as solvent instead of water, to shift the absorption band due to water bending to a part of the spectrum away from the amide I region. Deuterium oxide is usually employed instead of water because of its greater transparency in the region of interest. [220]

In this study the band resulting from the combination of bending and the libration mode of vibration of water molecules appears at around 2125 cm^{-1}. This was used to subtract the water from the dough spectrum following the approach according to the Dousseau method. [221] The complete removal of the band around 2125 cm^{-1} until a flat base line is obtained, also removes the band at 1640 cm^{-1}. As a result, the absorption bands in the amide I region are due only to the amide bond vibration modes. Therefore it can be seen from Figure 7-3 C

that the peak at around 2125 cm^{-1} becomes a flat line and the amide I and II became more fully resolved.

Another mode of water vibration is the libration mode of vibration, corresponding to a peak at around 800 cm^{-1} which is due to reorientation of the water molecules in the hydrogen bonded network. This is particularly sensitive to the dynamic properties of the water molecules. [310]

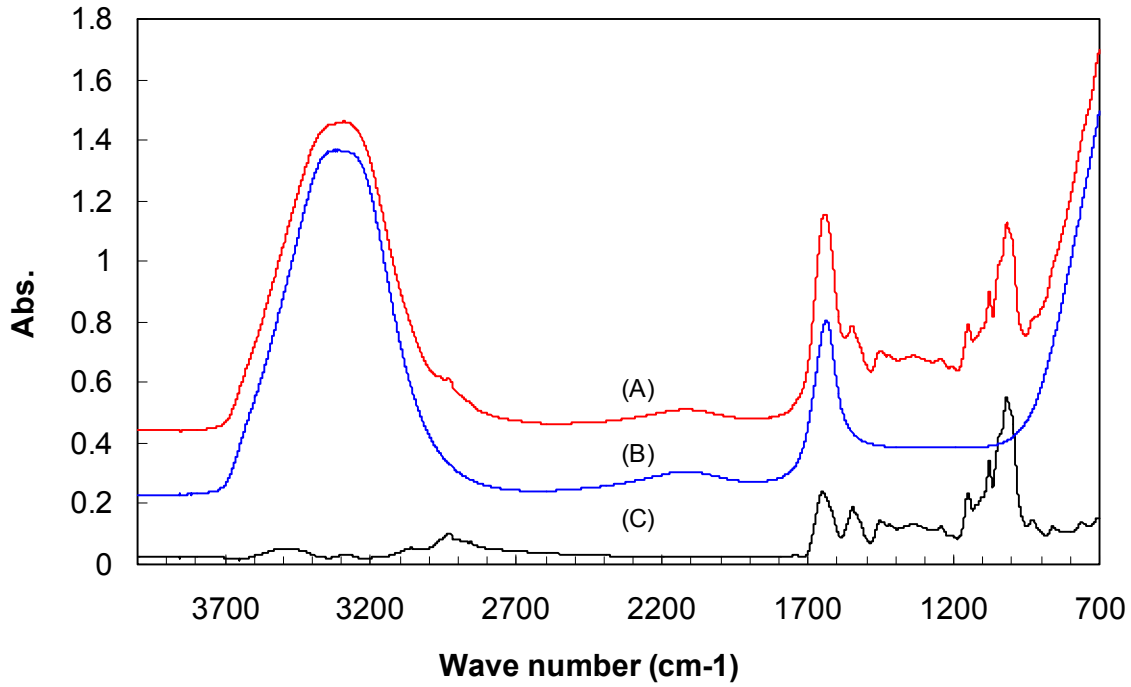

Figure 7-3 ATR/FT-IR spectra following deconvolution for (A) dough before water subtraction, (B) water, and (C) dough after water subtraction.

7.2.4 Deconvolution

As shown in Figure 7-2 ATR/FT-IR spectra of the food samples and in particular the protein amide I is composed of broad, overlapped bands. [309] Deconvolution is a resolution-enhancement procedure used to resolve overlapped and hidden peaks. Its application to ATR/FT-IR has made more detailed studies of globular proteins possible. [222] In the current study deconvolution was carried out by assuming an initial Gaussian [85, 223, 311] line-shape function for all the peaks. In order to measure the relative areas of the partially resolved amide I and II components the deconvoluted spectra were curve fitted. Peak fit version 4.12 software was used with the following settings: 63 filter, 9.57 and 0.05. The base line perfectly matched within the section of the spectrum at 880-2580 cm^{-1} and this was found to be a non-parametric base line with the data very closely matching the generated spectrum. During the current study, all of the data for all the experiments was treated in the

same way. Dividing the sum of the areas of all components associated with the given conformation, by the total amide I or II band, gives the relative area, which is used for the data analysis in this study. This approach has been widely used by other researchers. [224]

7.3 The effect of oxidising agent

This study monitors all of the changes in dough structure that are reflected by their vibration spectra. These include changes in protein secondary structure brought about by chemical additives at various levels of addition, while simultaneously reflecting the changes in starch regions as well as the amorphous and crystal structures of the dough spectrum. Figure 7-4 shows the effect of the addition of ascorbic acid at 300ppm level of addition on the dough.

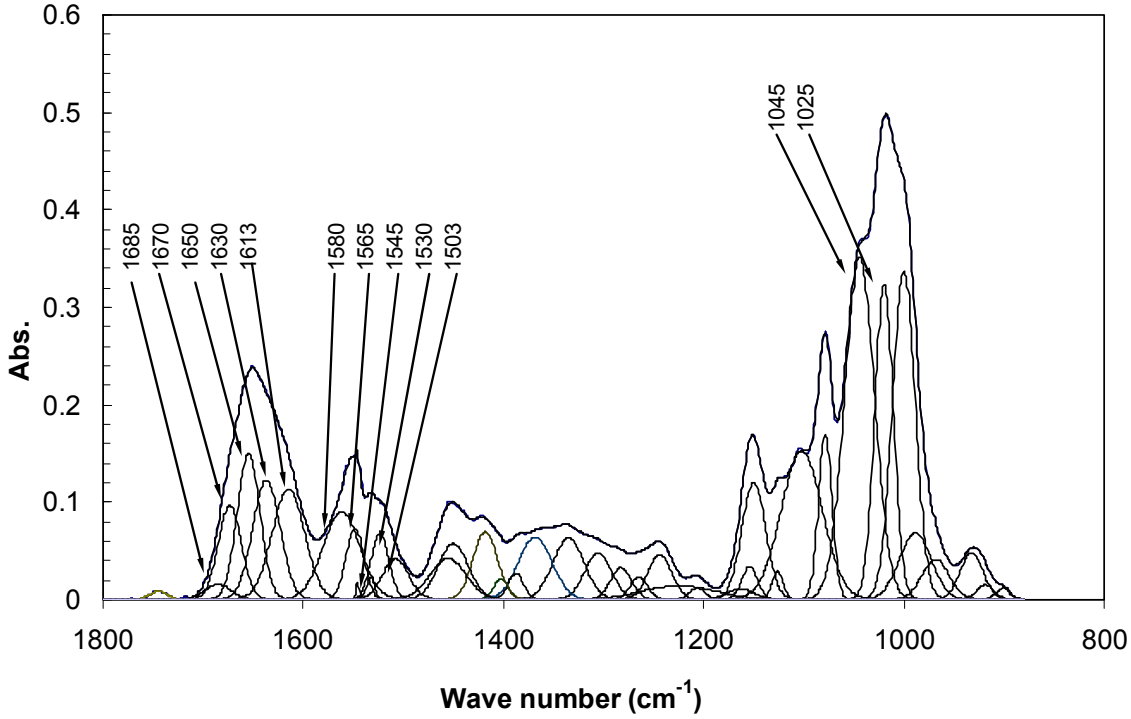

Figure 7-4 Wheat flour dough spectrum with 300ppm ascorbic acid following deconvolution

The addition of ascorbic acid to the dough results in an increase in the relative area of inter-molecular β-sheet (1613 cm^{-1}), while the intra-molecular β-sheet (1630 cm^{-1}) decreased. In Figure 7-7 to Figure 7-11 the increase in the inter-molecular β-sheet type of structure was due to the oxidation action of ascorbic acid on dough protein molecules, specifically the oxidation of sulfhydryl during the dough mixing process. Ascorbic acid is rapidly oxidised by atmospheric oxygen kneaded into the dough to give dehydroascorbic acid (DHA) and the reaction is accelerated by the presence of the enzyme ascorbate oxidase (AOX), naturally present in wheat flour. [91, 251, 312] The rate of this reaction during dough mixing is shown

in Figure 7-5. DHA is the cofactor of the enzyme glutathione dehydrogenase (GSH-DH) and this enzyme oxidises the glutathione (GSH) naturally present in wheat flour to produce disulfide linkages. [91, 312, 313] This reaction is linked to the baking quality of wheat flour dough. [314-316] The results obtained here will be further related to the baking results in the discussion presented in Chapter 7 of this thesis. The mechanism of the improver action of ascorbic acid is illustrated in Figure 7-6 and the possible reaction involved is presented in Table 7-1.

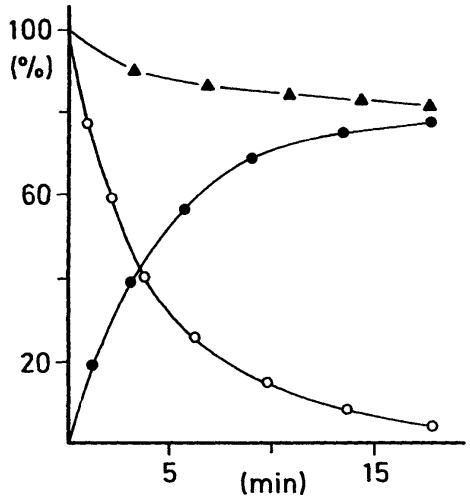

Figure 7-5 Oxidation of ascorbic acid to DHA during dough mixing [91]
○ Ascorbic acid ● DHA ▲ the sum of ascorbic acid and DHA

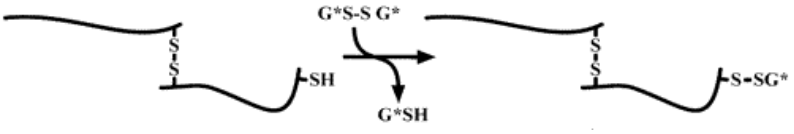

Figure 7-6 Representation of the improver action of ascorbic acid in the dough. [317]

The results indicate that the oxidation of sulfhydryl groups to the disulfide structure strengthens the protein- protein interactions and since glutenin polymers are richer in β-sheet structure than gliadins [188] the strengthening effect of ascorbic acid on the dough can be related to the amount and involvement of glutenin.

Table 7-1 The possible reactions involved in the improver action of ascorbic acid [317]

	Reactants	Catalyst	products
1	AA + ½ O_2	AOX	DHA + H_2O
2	DHA + 2GSH	GSH-DH	AA + GSSG
3	PSH + GSSG	→	PSSG + GSH
4	GSH + PSSP	→	GSSP + PSH
5	GSH + CSSC	→	GSSC + CSH
6	CSH + PSSP	→	CSSP + PSH
7	PSH + CSSC	→	PSSC + CSH

Note: GSSG, oxidised glutathione; CSSC, cystine; CSH, cysteine; PSSP, gluten; PSSG, gluten-bound glutathione; PSH, gluten sulfhydryl

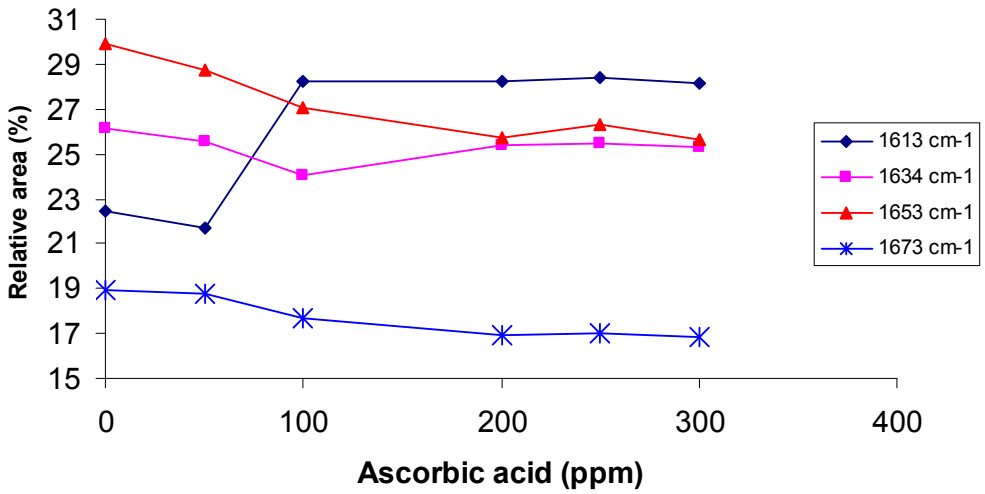

Figure 7-7 The effect of various levels of ascorbic acid on amide I components Note: Inter-molecular β-sheet 1613 cm^{-1}, Intra-molecular β-sheet 1630 cm^{-1}, α-helix 1653 cm^{-1}, and β-turn 1673 cm^{-1}

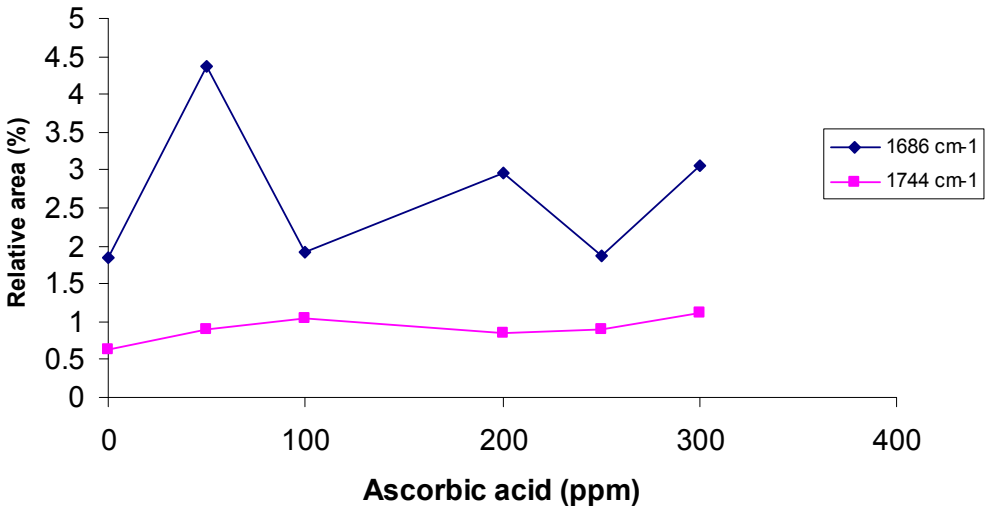

Figure 7-8 The effect of various levels of ascorbic acid on amide I components Antiparallel β-sheet 1686 cm^{-1}, and C=O of ascorbic acid 1744 cm^{-1}.

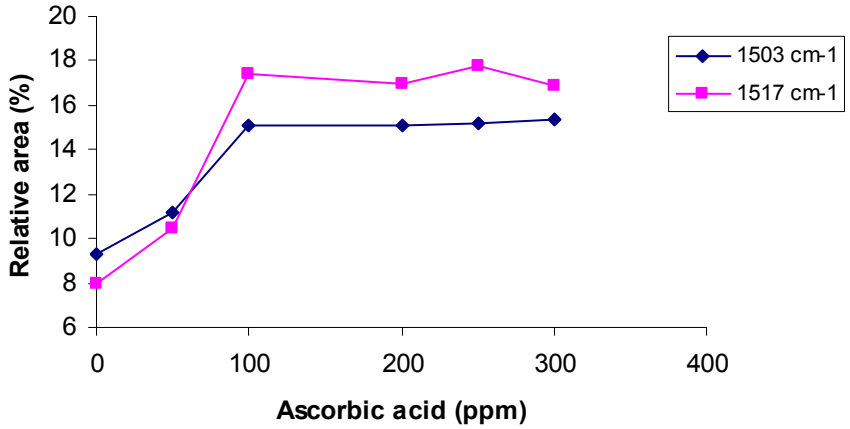

Figure 7-9 The effect of ascorbic acid on amide II components, the peaks at 1503 cm^{-1}, and 1517 cm^{-1}.

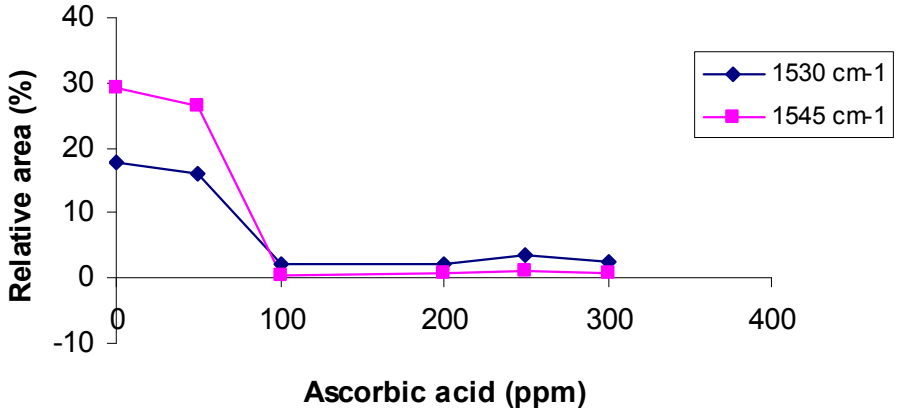

Figure 7-10 The effect of ascorbic acid on amide II components, the peaks at 1530 cm^{-1}, and 1545 cm^{-1}.

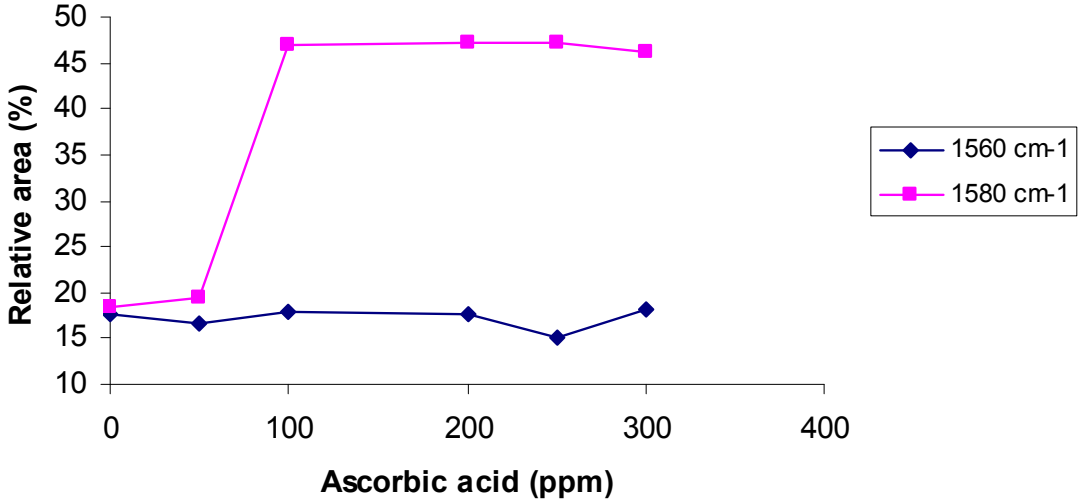

Figure 7-11 The effect of ascorbic acid on amide II components 1560 cm^{-1}, and 1580 cm^{-1}.

7.4 The effect of reducing agent

The addition of the reducing agent L-cysteine to wheat flour dough at various levels also has a substantial effect on the IR spectrum of the dough. Figure 7-12 shows the dough spectrum following deconvolution and peak fitting. Both amide I and amide II components have exhibited significant changes in relative area as a result of L-cysteine addition. These changes were the opposite of those observed when ascorbic acid were added. In addition,

the shape of both of the regions amide I and amide II has been affected by the addition of L-cysteine and as has the intensity of the different component of both amides.

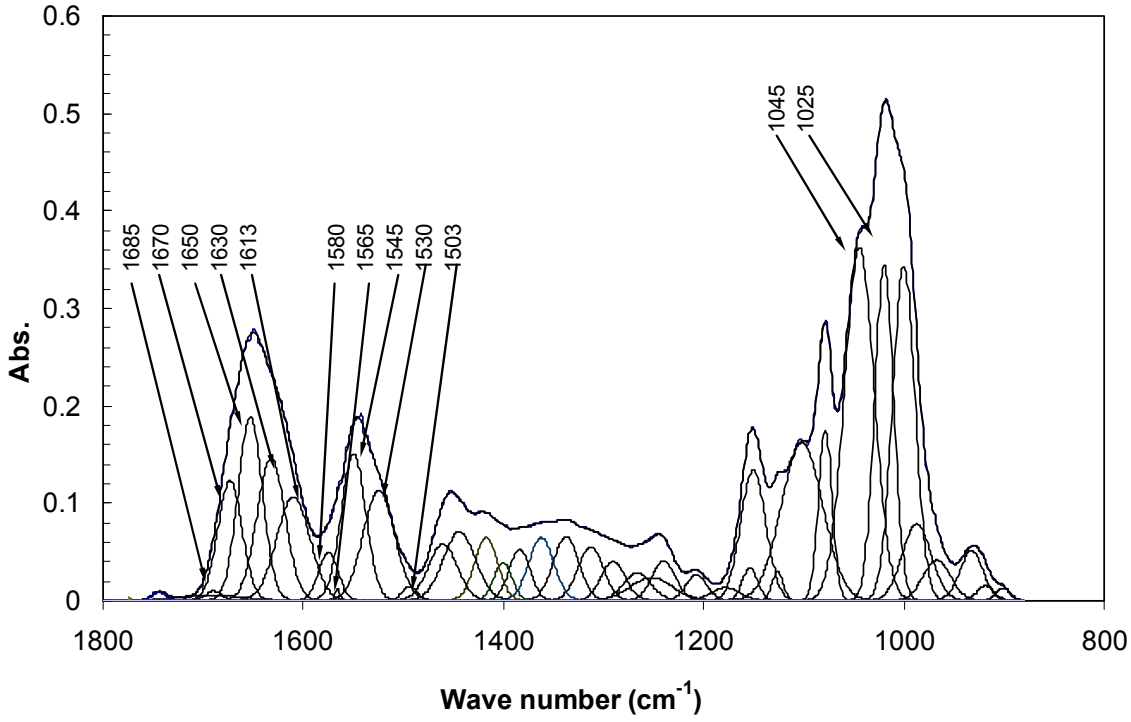

Figure 7-12 The spectrum of a wheat flour dough incorporating 15 ppm L-cysteine following deconvolution

L-Cysteine addition at levels of up to 10 ppm increased the relative area for both the inter-molecular β-sheet (1613 cm^{-1}), and intra-molecular β-sheet (1630 cm^{-1}) as shown in Figure 7-13 to Figure 7-16. The increases in the relative area were due to the reduction of disulfide bonds by L-cysteine, which results in increases in the protein-protein interactions within the same protein molecule (inter-molecular) and between one protein and other protein molecule (intra-molecular). The reduction of the disulfide bond results in an increase in the proportion of the protein having β-sheet structure in the dough. This corresponded with the dough becoming softer and easier to stretch, indicating that this type of secondary structure, when present, is characteristic of a weak dough with less resistance to extension. The addition of L-cysteine at levels of more than 10 ppm resulted in doughs being very sticky and soft: Handling of the dough became difficult because of the excessive cleavage of the disulfide bonds. The latter destroys the regularity of the protein secondary structure and the shape of the proteins becomes more symmetrical. The native macromolecules of the protein can be strained because of the chemical bridges connecting different parts of its main valency chain. [318]

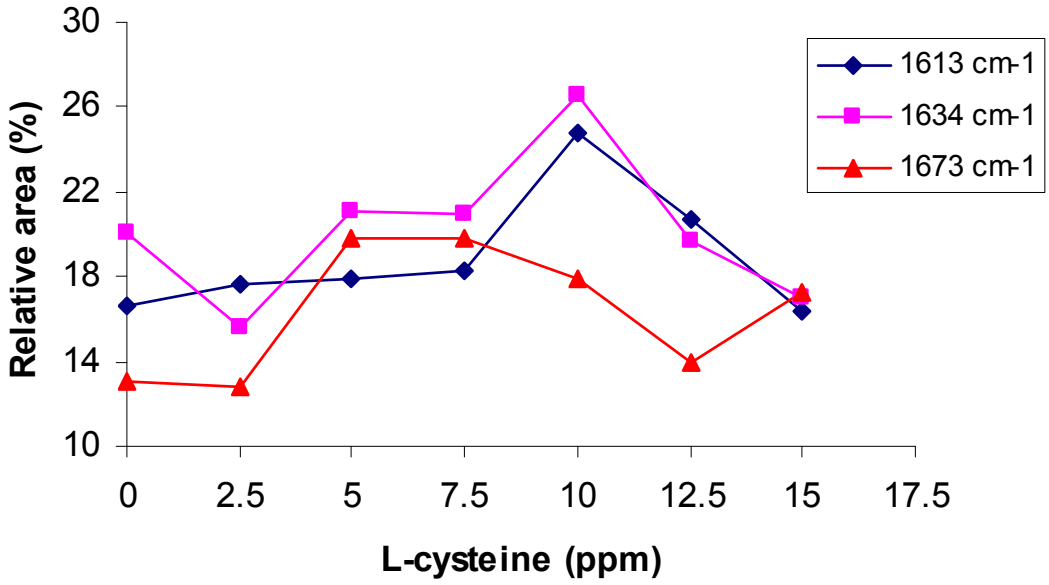

Figure 7-13 The effect of various levels of L-cysteine on amide I components. Inter-molecular β-sheet 1613 cm^{-1}, Intra-molecular β-sheet 1634 cm^{-1}, and β-turn 1673 cm^{-1}

While the β- sheet structure increased, the presence of the band due to the α-helix structure exhibited substantial decreases in relative area (Figure 7-14 toFigure 7-16) as a result of the reduction of the disulfide bond in glutenin. This indicates that the α-helix structure is relatively rigid and less accessible to water compared to the other amide I components including β-sheet while also being more hydrophobic. The reduction of the disulfide bond reduces the size of the gluten molecules by allowing separation of the peptide chains. This reduces the distortion of the polypeptide backbones which is at least partially responsible for the presence of the helical structure. [189]

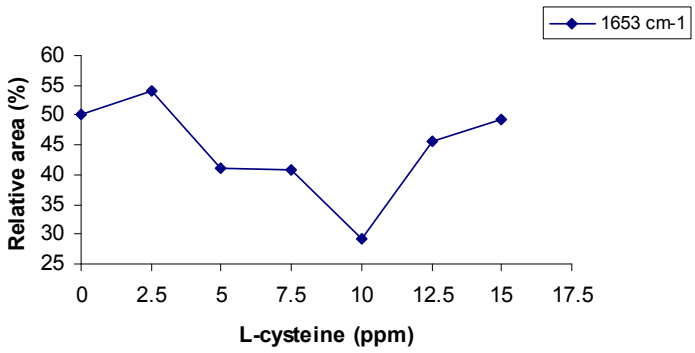

Figure 7-14 The effect of various levels of L-cysteine on α-helix (1653 cm^{-1})

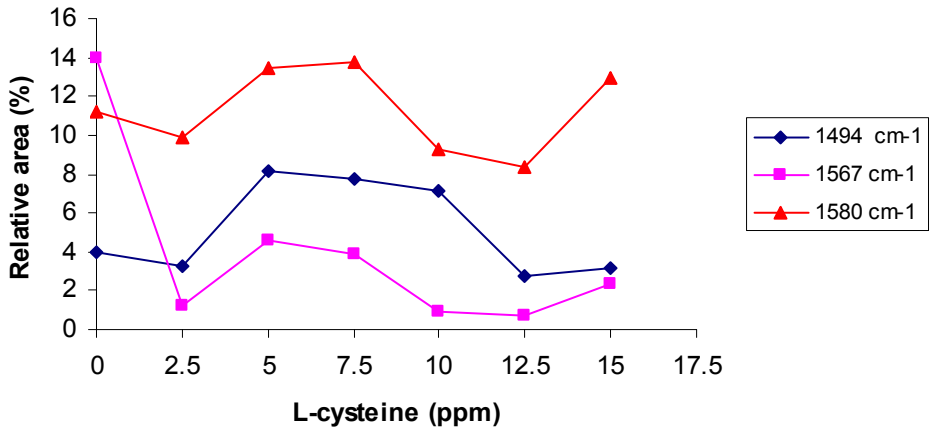

Figure 7-15 Effect of various levels of L-cyteine on amide II components (1494 cm^{-1}, 1567 cm^{-1}, and 1580 cm^{-1})

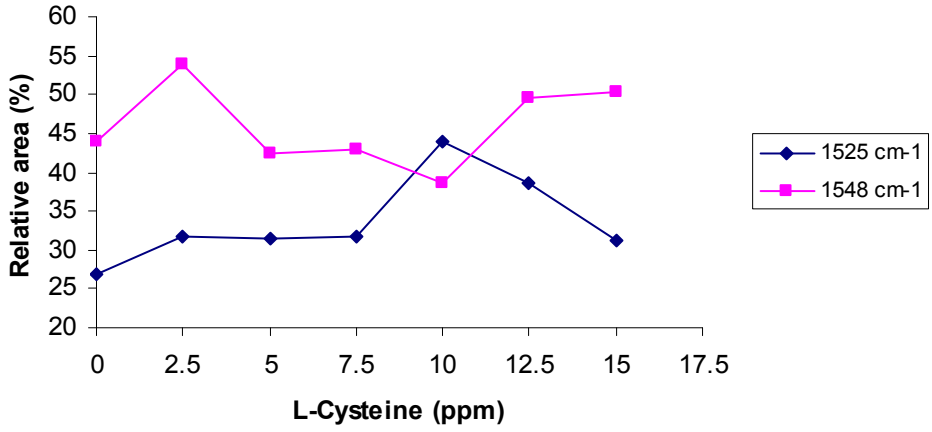

Figure 7-16 Effect of various levels of L-cyteine on amide II components (1525 cm^{-1} and 1548 cm^{-1})

7.5 Conclusion

The modifications which were observed to occur in the chemical structure of the protein as a result of the action of the food additives, corresponded with significant changes in the rheological properties of the wheat flour doughs. This has been confirmed by the baking experiments which will be discussed in Chapter 7 of this thesis which show that the changes in protein secondary structure affect the baking performance of wheat flour dough.

From the studies reported in this chapter, FT-IR has been shown to provide an effective means to monitor the protein-protein interactions, starch-protein interactions and disulfide

sulfhydryl interchange. These are the main elements that determine the overall dough strength and therefore, processing characteristics of the dough.

8

8 Conclusions and recommendations for future research

8.1 Starch and α-amylase reaction

Throughout this study the technique of ATR/FT-IR has been utilised in studies of dough processing and baking. The results clearly demonstrate that this is a useful approach which can enhance our understanding of the changes occurring in a dough. ATR/FT-IR has been applied to estimating and evaluating a number of components having significance in the bread making process.

Firstly the technique has been utilised for the investigation of starch hydrolysis and also to the qualitative measurement of sugars within the dough system. The kinetics of α-amylase reaction with starch has been considered in two ways: by monitoring both the rate of production of sugars in the reaction medium as well as by assessing the cleavage of the glycosidic linkages in starch. The findings were that when various ingredients were incorporated and the impact of processing conditions was evaluated, it was possible to determine their impact upon the kinetics of the cleavage of the glycosidic linkage. These effects could be readily monitored. Accordingly, the effects of dough ingredients on starch were clearly seen. Correspondingly, structural changes in starch were monitored and these were shown to be directly related to the period of time which had elapsed for the incubation of starch in the dough with the α-amylase. These observations were also related to the intra- and inter-molecular interactions accompanying the reaction catalysed by the enzyme α-amylase. The various quality attributes of the end product in the form of bread loaves produced from the doughs were measured and these included loaf volumes, crumb texture and colour characteristics. The quality of the resultant loaves were strongly influenced by the action of the enzyme and it was possible to effectively monitor the whole fermentation process using ATR/FT-IR.

8.2 Protein secondary structure

Extensive research has previously been carried out on the role of protein components in the formation of the gluten matrix and their significance in the development of desirable dough characteristics for bread making. In this context there have been relatively few studies that have been able to evaluate the relative significance of the recognised forms of protein secondary structure. In addition, whilst it is recognised that bakery additives in the form of dough conditioners can be used to manipulate dough and bread characteristics, the application of ATR/FT-IR instrumentation to monitor these has not been reported.

A number of chemical additives significantly change the rheological properties of wheat flour doughs and the baking experiments discussed in chapter 7 have significantly advanced our knowledge of the use of ATR/FT-IR as a tool in this area of research. Changes in protein secondary structure clearly affect the baking performance of wheat flour doughs. The addition of the oxidising agent AsA results in a decrease in the relative area of the IR peaks that reflect the interactions of the structure identified as reflecting the formation and presence of inter-molecular β-sheets. The results indicate that the oxidation of sulfhydryl groups to form disulfide linkages strengthens the protein-protein interactions. Since glutenin polymers are known to be higher in β-sheet structures than gliadins, the results obtained in the current study provide confirm that the strength effect shown to be associated with AsA incorporation into the dough formulation are directly related to role of the glutenin component of the gluten-forming proteins.

The results obtained in the current study are consistent with the developing understanding that the overall strength of wheat flour doughs reflects the significance of three main elements which are protein-protein interactions, starch-protein interactions as well as disulfide-sulfhydryl interchange. ATR/FT-IR provides a useful means of comparing doughs and studying the changes occurring during dough development and also as a result of subsequent processing.

8.3 ATR/FT-IR and the interactions occurring during baking

Following the studies of doughs, a similar approach has been applied for a re-evaluation of the interactions and the synergetic effect of ingredients during baking. Consistent with the accumulated knowledge of baking systems, it has been demonstrated here that the incorporation of α-amylase increases bread volume, and also serves to retard bread staling while the addition of the reducing agent L-cysteine increases bread volume up until a certain point has been reached, followed by a clear decrease in bread volume. Sodium chloride

levels in the baking formulation were shown to influence yeast growth and α-amylase action on starch. The results show that, based upon the quality attributes measured in this study, the optimum level of salt incorporation was found to be within the range of 1.6-1.8% based on flour weight.

It was found that within the wheat flour doughs, the preparation with the addition of the oxidising agent and reducing agent, both had a marked effect upon dough protein secondary structure. This then affected the baking performance as reflected in the measured attributes of baked loaves.

8.4 Recommendations for future research into wheat flour dough

It is generally accepted that wheat flour doughs are complex matrices and that a diverse range of molecular components are probably interacting, thereby influencing the quality of the products ultimately consumed as part of the diet throughout the world. Whilst the current investigation provides novel results and establishes the potential and usefulness of the approaches developed here, much remains to be done to extend the current results. Among the additional work that can now be considered, the use of ATR/FT-IR warrants further attention.

A number of components and interactions that were beyond the scope of this research project, can now be considered as part of ongoing and future research. This has the potential to extend the applications and usefulness of ATR/FT-IR in the processing of a variety of foods made from cereal grains.

Firstly further investigation into the determination of the starch damage content of wheat flour is recommended as the preliminary evidence reported here is promising. The peak identified as being useful for this purpose and corresponding to damage starch in wheat flour doughs is the peak occurring at 975 cm^{-1}. During the current study this peak has been monitored in conjunction with the assessment of the effect α-amylase on the relative area of this peak. Clearly, future quantitative measurement of damaged starch should focus on this peak.

Another area recommend for ongoing studies is related to the effects of various levels of addition of different baking ingredients. It is suggested that the impact of a range of bakery ingredients be analysed regarding protein secondary structure as well as the activity of α-amylase. Based upon the results reported in this thesis, the determination of α-amylase activity in wheat flour by using ATR/FT-IR appears to be achievable. This might be done

either by monitoring the rate of production of the various sugar products in the dough spectra, or alternatively by monitoring the cleavage of the glycosidic linkage. It will be useful to compare these two approaches as a means of determining their relative suitability for future adoption in routine analysis that might provide useful strategies for quality control and assurance.

On the basis of the demonstration of the potential of vibrational spectroscopy instruments for the study of wheat flour doughs, further work on disulfide interactions and their significance are also recommended. Instruments including ATR/FT-IR as well as Raman spectroscopy may well provide invaluable tools and information. In future studies the estimation of the disulfide content of wheat flour dough might be explored. During the current work, both the disulfide and sulfhydryl peaks appeared to be relatively weak in ATR/FT-IR. However these may be more readily detected in Raman spectroscopy. Accordingly research in the area of the disulfide-sulfhydryl interactions might usefully focus on the use of Raman spectroscopy rather than using ATR/FT-IR. In the context of increasing evidence of the importance of selenium in wheat flour doughs, along with the likelihood of links forming between the seleno-amino acids, particularly selenocysteine in doughs, it is also possible that Raman spectroscopy may be applied to this area as well.

8.5 Recommendations for future research in food products

The results from this study demonstrating that computer software that is able to effect deconvolution of IR spectra obtained for dough samples, indicates that it may also be possible to use this approach for other measurements. These may usefully include the determination of a range of other components found in flours and doughs, as well as extending to constituents of other grains and foods more generally.

As examples, the determination of ascorbic acid content in wheat flour and other food products can probably be achieved by monitoring the vibration corresponding with C=O stretch appearing at 1743cm^{-1} in the spectrum. In the study reported in this thesis, the peak of this particular vibration is strong in the ATR/FT-IR spectra. In addition, the relative area of this peak has been shown to have increased with increasing levels of addition of AsA in wheat flour doughs.

The focus of this thesis has been upon the various peaks and aspects of starch and protein components. In addition to these, it is likely that ATR/FT-IR can be evaluated and used to study other food constituents including lipids and the non-starch polysaccharides in doughs, the resultant products and other foods.

Another area of interest is in the peak identified as corresponding to O-H stretch specifically of water at around 2800-3800 cm^{-1}. This relates to the degree of hydration of the samples and to that of the various components in foods which have varying affinities for water. Based upon the observations made here, it is also likely that ATR/FT-IR can prove useful to future studies of the hydration kinetics of food samples. It might be expected that this peak can be used to study protein-water and starch-water interactions. Other groups of food components are also involved in the competition for hydration. This region of the spectrum may provide promising insights through the monitoring of changes occurring during processing and storage.

Finally, whilst the results discussed here have necessarily been limited in scope, they do demonstrate the potential of the approach described. Further work is now warranted and it is sincerely hoped that what has already been achieved might stimulate further studies in this area, thereby enhancing our understanding of dough systems as well as other foods, and that this might ultimately lead to more effective ways of providing nutritious and appealing food products in the future.

9 References

1. Singh, H., *A study of changes in wheat protein during bread baking using SE-HPLC.* Food Chem., 2004. **90**(1-2): p. 247-250.
2. Rosell, C.M., J.A. Rojas, and C. Benedito de Barber, *Influence of hydrocolloids on dough rheology and bread quality.* Food Hydrocolloids, 2001. **15**(1): p. 75-81.
3. Romano, A., et al., *Description of leavening of bread dough with mathematical modelling.* Journal of Food Engineering, 2007. **83**(2): p. 142-148.
4. Scanlon, M.G. and M.C. Zghal, *Bread properties and crumb structure.* Food Research International, 2001. **34**(10): p. 841-864.
5. Chin, N.L., P.J. Martin, and G.M. Campbell, *Aeration During Bread Dough Mixing: I. Effect of Direction and Size of a Pressure Step-change During Mixing on the Turnover of Gas.* Food and Bioproducts Processing, 2004. **82**(4): p. 261-267.
6. Martin, P.J., et al., *Aeration During Bread Dough Mixing: III. Effect of Scale-up.* Food and Bioproducts Processing, 2004. **82**(4): p. 282-290.
7. Shimiya, Y. and T. Yano, *Measurements of accompanying air, specific surface area and micropore volume of some flour particles.* Agric. Biol. Chem., 1987. **51**(1): p. 25-30.
8. Örnebro, J., T. Nylander, and A.C. Eliasson, *Interfacial Behaviour of Wheat Proteins.* J. Cereal Sci., 2000. **31**(2): p. 195-221.
9. Every, D., et al., *Predicting wheat quality - consequences of the ascorbic acid improver effect.* J. Cereal Sci, 2008. **48**(2): p. 339-348.
10. Vaclavik, V.A. and E.W. Christian, *Fat and Oil Products*, in *Essentials of Food Science*. 2008. p. 273-309.
11. Gould, J.T., *Baking Around the World*, in *Technology of Breadmaking*. 2007. p. 223-243.
12. Vaclavik, V.A. and E.W. Christian, *Baked Products: Batters and Dough*, in *Essentials of Food Science*. 2008. p. 351-378.
13. Cauvain, S.P., *Breadmaking Processes*, in *Technology of Breadmaking*. 2007. p. 21-49.
14. Kuktaite, R., H. Larsson, and E. Johansson, *The Influence of Dough Mixing Time on Wheat Protein Composition and Gluten Quality for Four Commercial Flour Mixtures*, in *Wheat Production in Stressed Environments*, H.T. Buck, J.E. Nisi, and N. Salomón, Editors. 2007, Springer Netherlands. p. 543-548.
15. Chiotellis, E. and G.M. Campbell, *Proving of Bread Dough II: Measurement of Gas Production and Retention.* Food and Bioproducts Processing, 2003. **81**(3): p. 207-216.
16. Shah, P., et al., *Proving of Bread Dough: Modelling the Growth of Individual Bubbles.* Food and Bioproducts Processing, 1998. **76**(2): p. 73-79.
17. io.uwinnipeg.ca/~simmons/16cm05/1116/16fungi.htm.
18. Mills, E.N.C., et al., *Bubble Formation and Stabilization in Bread Dough.* Food and Bioproducts Processing, 2003. **81**(3): p. 189-193.
19. Mondal, A. and A.K. Datta, *Bread baking - A review.* Journal of Food Engineering, 2008. **86**(4): p. 465-474.
20. Zhang, L., et al., *Effects of crust constraints on bread expansion and CO2 release.* Journal of Food Engineering, 2007. **80**(4): p. 1302-1311.
21. Fan, J., J.R. Mitchell, and J.M.V. Blanshard, *A model for the oven rise of dough during baking.* Journal of Food Engineering, 1999. **41**(2): p. 69-77.
22. Hoseney et al., H., R.C., Sievert, D., Delcour, J.A.,, *Bread and other baked products*, in *Ullmann's Encyclopedia of Industrial Chemistry*. 2008, Wiley-VCH Verlag: Weinheim, Germany.

23. Therdthai, N., W. Zhou, and T. Adamczak, *Optimisation of the temperature profile in bread baking.* Journal of Food Engineering, 2002. **55**(1): p. 41-48.
24. Yamada, Y. and K.R. Preston, *Effect of individual oxidants on oven rise and bread properties of Canadian short process bread.* J. Cereal Sci., 1992. **15**(3): p. 237-51.
25. Stathopoulos, C.E., et al., *Effect of heat on rheology of gluten fractions from flours with different bread-making quality.* J. Cereal Sci., 2006. **43**(3): p. 322-330.
26. Patel, B.K., R.D. Waniska, and K. Seetharaman, *Impact of different baking processes on bread firmness and starch properties in breadcrumb.* J. Cereal Sci, 2005. **42**(2): p. 173-184.
27. Bloksma, A.H., *Effect of heating rate on viscosity of wheat flour doughs.* Journal of Texture Studies, 1980. **10**(3): p. 261-269.
28. Doublier, J.L., G. Llamas, and M. Le Meur, *A rheological investigation of cereal starch pastes and gels. Effect of pasting procedures.* Carbohydrate Polymers, 1987. **7**(4): p. 251-275.
29. Wootton, M. and A. Bamunuarachchi, *Application of Differential Scanning Calorimetry to Starch Gelatinization. II. Effect of Heating Rate and Moisture Level.* Starch - Stärke, 1979. **31**(8): p. 262-264.
30. Eliasson, A.C., *Effect of Water Content on the Gelatinization of Wheat Starch.* Starch - Stärke, 1980. **32**(8): p. 270-272.
31. Goesaert, H., et al., *Amylases and bread firming - an integrated view.* J. Cereal Sci., 2009. **50**(3): p. 345-352.
32. Jeanjean, M.F., R. Damidaux, and P. Feillet, *Effect of heat treatment on protein solubility and viscoelastic properties of wheat gluten.* Cereal Chem., 1980. **57**(5): p. 325-31.
33. Morel, M.-H., A. Redl, and S. Guilbert, *Mechanism of Heat and Shear Mediated Aggregation of Wheat Gluten Protein upon Mixing.* Biomacromolecules, 2002. **3**(3): p. 488-497.
34. Schofield, J.D., et al., *The effect of heat on wheat gluten and the involvement of sulfydryl-disulfide interchange reactions.* J. Cereal Sci., 1983. **1**(4): p. 241-53.
35. Weegels, P.L., et al., *Effects on gluten of heating at different moisture contents. II. Changes in physicochemical properties and secondary structure.* J. Cereal Sci., 1994. **19**(1): p. 39-47.
36. Purlis, E. and V.O. Salvadori, *Bread browning kinetics during baking.* Journal of Food Engineering, 2007. **80**(4): p. 1107-1115.
37. Purlis, E. and V.O. Salvadori, *Modelling the browning of bread during baking.* Food Research International, 2009. **42**(7): p. 865-870.
38. Zanoni, B., C. Peri, and D. Bruno, *Modelling of browning kinetics of bread crust during baking.* Lebensmittel-Wissenschaft und-Technologie, 1995. **28**(6): p. 604-609.
39. Vanin, F.M., T. Lucas, and G. Trystram, *Crust formation and its role during bread baking.* Trends in Food Science & Technology, 2009. **20**(8): p. 333-343.
40. Wählby, U. and C. Skjöldebrand, *Reheating characteristics of crust formed on buns, and crust formation.* Journal of Food Engineering, 2002. **53**(2): p. 177-184.
41. Wagner, M.J., et al., *Water transport in bread during baking.* Journal of Food Engineering, 2007. **78**(4): p. 1167-1173.
42. Whitford, D., *Proteins: Structure and Function*. 2005: John Wiley & Sons. 528.
43. http://bill.srnr.arizona.edu/classes/182/PeptideBond-HiRes.JPEG.
44. *Amino Acids, Peptides, Proteins*, in *Food Chem*. 2009. p. 8-92.
45. Dzwolak, W., M. Kato, and Y. Taniguchi, *Fourier transform infrared spectroscopy in high-pressure studies on proteins.* Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 2002. **1595**(1-2): p. 131-144.
46. Vaclavik, V.A. and E.W. Christian, *Proteins in Food: An Introduction*, in *Essentials of Food Science*. 2008. p. 145-159.
47. Anon, *Instant notes: biochemistry (3rd ed.) by Hames, David and Hooper*. Biochem. Mol. Biol. Educ. . Vol. 34. 2006. 59-60.
48. Murray, R.K. and et al., *Harper's Biochemistry*. 24th Edition ed. 1996. 868 pp.
49. https://eapbiofield.wikispaces.com/Katie+Chapter+2?f=print.

50. http://en.wikibooks.org/wiki/Biochemistry/Proteins/The_chemistry_of_proteins.
51. Silberberg, M., *Chemistry: The Molecular Nature of Matter and Change; 3rd Edition*. 2002. 1062.
52. Kilgar, G.L., *Fundamentals of Biochemistry*. 1981. 380 pp.
53. Chen, H. and J. Skolnick, *M-TASSER: an algorithm for protein quaternary structure prediction.* Biophys. J., 2008. **94**(3): p. 918-928.
54. Bhagavan, N.V., *Three-Dimensional Structure of Proteins*, in *Medical Biochemistry*. 2002, Academic Press: San Diego. p. 51-65.
55. http://www.moleculesinmotion.com/protarch/page_helix/intro.html.
56. Murray, R.K., et al., *Harper's Biochemistry* 24th ed. Lange Series. 1996: Appleton & Lange. 820pp.
57. Holum, J.R., *Fundamentals of General, Organic, and Biological Chemistry, 6th Edition*. 1997. 880 pp.
58. Yu, X., C. Wang, and Y. Li, *Classification of protein quaternary structure by functional domain composition.* BMC Bioinf., 2006. **7**: p. No pp given.
59. http://academic.brooklyn.cuny.edu/biology/bio4fv/page/3d_prot.htm.
60. Greenwood, N.N. and A. Earnshaw, *Chemistry of the Elements*. 2nd Edition ed. 1997. 1542.
61. http://hyperphysics.phy-astr.gsu.edu/hbase/chemical/bond.html#c7.
62. http://www.microbelibrary.org/index.asp.
63. Ovaa, H. and A.N.M. Schumacher, *Breaking noncovalent binding interactions in multimeric proteins: dissociation of MHC-bound ligand through the use of light-sensitive or periodate sensitive group*. 2006, (Het Nederlands Kanker Instituut, Neth.). Application: EP
EP. p. 19 pp.
64. Gaonkar, A.G., A. McPherson, and Editors, *Ingredient Interactions: Effects on Food Quality, Second Edition. [In: Food Sci. Technol. (Boca Raton, FL, U.S.); 2006, 154]*. 2006. 554 pp.
65. Rao, M.V.R., M. Atreyi, and M.R. Rajeswari, *Specific interactions between amino acid side chains - a partial molar volume study.* Can. J. Chem., 1988. **66**(3): p. 487-90.
66. Smith, B.C., *Infrared Spectral Interpretation: A Systematic Approach*. 1999. 200 pp (approx).
67. George, W.O. and P.S. Mcintyre, *infrared Spectroscopy*. Analytical chemistry by open learning. 1987, New York: John Wiley &Sons. 537.
68. http://www.bbemg.ulg.ac.be/Images/UKondeelm.gif.
69. Hester, R., *Handbook of Vibrational Spectroscopy, John Chalmers & Peter Griffiths (eds)*. Chem. Ind. (London, U. K.). 2002. 23-24.
70. Harris, D.C. and M.D. Bertolucci, *Symmetry and Spectroscopy. An Introduction to Vibrational and Electronic Spectroscopy*. 1989. 550 pp.
71. http://physics.schooltool.nl/irspectroscopy/theory.php.
72. Smith, A.L., *Applied Infrared Spectroscopy: Fundamentals, Techniques, and Analytical Problem Solving*. 1979. 322 pp.
73. http://atropos.as.arizona.edu/aiz/teaching/a204/lecture1.html.
74. http://www.specialtyinspections.com/images/electro_clip_image003.gif.
75. Skoog, D.A., D.M. West, and F.J. Holler, *Fundamentals of Analytical Chemistry. 5th Ed*. 1988. 894 pp.
76. Calton, E.F., *Fourier Transform Infrared Spectrometry. 2nd Edition. P. R. Griffiths and J. A. de Haseth, Eds*. Appl. Spectrosc. . Vol. 61. 2007. 289A.
77. Griffiths, P.R., *Fourier transform infrared spectrometry.* Science (N.Y.), 1983. **222**(4621): p. 297-302.
78. Griffiths, P.R., *Fourier Transform Infrared Spectrometry: Theory and Instrumentation*, in *Transform Techniques in Chemistry*, P.R. Griffiths, Editor. 1978, Plenum Publishing Corporation: New York. p. 109-39.
79. Davis, S.P., M.C. Abrams, and J.W. Brault, *Fourier analysis*, in *Fourier Transform Spectrometry*. 2001, Academic Press: San Diego. p. 41-66.

80. Nakamoto, K. and Editor, *Infrared and Raman Spectra of Inorganic and Coordination Compounds, Part A: Theory and Applications in Inorganic Chemistry, Sixth Edition*. 2009. 419 pp.
81. Davis, S.P., M.C. Abrams, and J.W. Brault, *Introduction*, in *Fourier Transform Spectrometry*. 2001, Academic Press: San Diego. p. 1-16.
82. http://spectroscopy.lbl.gov/FTIR-Martin/.
83. http://www.dliengineering.com/vibman/gloss_modeofvibration1.htm.
84. Durig, J.R., *NATO Advanced Study Institute Series: Series C, Vol. 57: Analytical Applications of FT-IR to Molecular and Biological Systems [Proceedings of the NATO Advanced Study Institute Held at Florence, Italy, August 31 to September 12, 1979]*. 1980. 607 pp.
85. Barth, A., *Infrared spectroscopy of proteins.* Biochim. Biophys. Acta, Bioenerg., 2007. **1767**(9): p. 1073-1101.
86. Goormaghtigh, E., V. Raussens, and J.-M. Ruysschaert, *Attenuated total reflection infrared spectroscopy of proteins and lipids in biological membranes.* Biochim. Biophys. Acta, Rev. Biomembr., 1999. **1422**(2): p. 105-185.
87. http://www.spectroscopynow.com/coi/cda/detail.cda?id=18427&type=EducationFeature&chId=2&page=1.
88. Davila-El Rassi, G.M., et al., *Studies of molecular association of glutenins and maltodextrin in solution by capillary electrophoresis.* J. Liq. Chromatogr. Relat. Technol., 2003. **26**(15): p. 2465-2479.
89. Roberts, T.A., et al., *Cereals and cereal products: Micro-Organisms in Foods 6*, in *Micro-Organisms in Foods 6*, T.A. Roberts, et al., Editors. 2005, Springer US. p. 392-439.
90. Autran, J.C., *Recent perspectives on the genetics, biochemistry and functionality of wheat proteins.* Trends in Food Science & Technology, 1993. **4**(11): p. 358-64.
91. Belitz, H.-D., W. Grosch, and P. Schieberle, *Cereals and Cereal Products*, in *Food Chemistry*. 2009, Springer: Berlin p. 670-745.
92. Aminlari, M. and M. Majzoobi, *Effect of chemical modification, pH change, and freezing on the rheological, solubility, and electrophoretic pattern of wheat flour proteins.* J. Food Sci., 2002. **67**(7): p. 2502-2506.
93. Sievert, D. and R.C. Hoseney, *Wheat Types and their Uses* in *Bread and Other Baked Products*. 2005, Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim. p. 2.
94. Martin, D.J. and B.G. Stewart. in *ASEAN Food J*. 1994.
95. Matz, s.A., *Wheat Ingredients*, in *Bakery Technology and Engineering*, s.A. Matz, Editor. 1992, Van Nostrand Reinhold: New York. p. 3.
96. James, M.G., K. Denyer, and A.M. Myers, *Starch synthesis in the cereal endosperm.* Current Opinion in Plant Biology, 2003. **6**(3): p. 215-222.
97. Manners, D.J., *Recent developments in our understanding of amylopectin structure.* Carbohydr. Polym., 1989. **11**(2): p. 87-112.
98. Copeland, L., et al., *Form and functionality of starch.* Food Hydrocolloids, 2009. **23**(6): p. 1527-1534.
99. Zobel, H.F., *Molecules to granules: a comprehensive starch review.* Starch/Staerke, 1988. **40**(2): p. 44-50.
100. Hannah, L., *Starch Formation in the Cereal Endosperm*, in *Endosperm*. 2007. p. 179-193.
101. Goesaert, H., et al., *Wheat flour constituents: how they impact bread quality, and how to impact their functionality.* Trends in Food Science & Technology, 2005. **16**(1-3): p. 12-30.
102. Gibson, T.S., V.A. Solah, and B.V. McCleary, *A procedure to measure amylose in cereal starches and flours with concanavalin A.* J. Cereal Sci., 1997. **25**(2): p. 111-119.
103. Lindeboom, N., P.R. Chang, and R.T. Tyler, *Analytical, biochemical and physicochemical aspects of starch granule size, with emphasis on small granule starches: a review.* Starch/Staerke, 2004. **56**(3-4): p. 89-99.

104. Jenkins, P.J. and A.M. Donald, *Gelatinisation of starch: a combined SAXS/WAXS/DSC and SANS study.* Carbohydrate Research, 1998. **308**(1-2): p. 133-147.
105. Perry, P.A. and A.M. Donald, *The effect of sugars on the gelatinisation of starch.* Carbohydr. Polym., 2002. **49**(2): p. 155-165.
106. Crochet, P., et al., *Starch crystal solubility and starch granule gelatinisation.* Carbohydrate Research, 2005. **340**(1): p. 107-113.
107. Parker, R. and S.G. Ring, *Aspects of the Physical Chemistry of Starch.* J. Cereal Sci, 2001. **34**(1): p. 1-17.
108. Evers, A.D. and D.J. Stevens, *Production and measurement of starch damage in flour. Part 4: Effect of starch damage on hot pasting properties.* Starch/Staerke, 1988. **40**(8): p. 297-9.
109. Weegels, P.L., R.J. Hamer, and J.D. Schofield, *Functional properties of wheat glutenin.* J. Cereal Sci., 1996. **23**(1): p. 1-18.
110. Shewry, P.R., et al., *The structure and properties of gluten: An elastic protein from wheat grain.* Philos. Trans. R. Soc. London, Ser. B, 2002. **357**(1418): p. 133-142.
111. Song, Y. and Q. Zheng, *Dynamic rheological properties of wheat flour dough and proteins.* Trends in Food Science & Technology, 2007. **18**(3): p. 132-138.
112. Wellner, N., et al., *Effect of selected hofmeister anions on the secondary structure and dynamics of wheat prolamins in gluten.* Cereal Chem., 2003. **80**(5): p. 596-600.
113. Shewry, P.R. and A.S. Tatham, *Disulfide bonds in wheat gluten proteins.* J. Cereal Sci., 1997. **25**(3): p. 207-227.
114. Stauffer, C.E., *Principles of Dough Formation* in *Technology of Breadmaking* S.P.C.a.L.S. Young, Editor. 2007, Springer US. p. 299-332.
115. Wieser, H., *Chemistry of gluten proteins.* Food Microbiol., 2007. **24**(2): p. 115-119.
116. Khatkar, B.S., et al., *Functional Properties of Wheat Gliadins. II. Effects on Dynamic Rheological Properties of Wheat Gluten.* J. Cereal Sci., 2002. **35**(3): p. 307-313.
117. Khatkar, B.S., A.E. Bell, and J.D. Schofield, *The dynamic rheological properties of glutens and gluten sub-fractions from wheats of good and poor bread making quality.* J. Cereal Sci., 1995. **22**(1): p. 29-44.
118. Sun, S., Y. Song, and Q. Zheng, *Rheological behavior of heat-induced wheat gliadin gel.* Food Hydrocolloids, 2008. **23**(3): p. 1054-1056.
119. McCann, T.H., et al., *Protein-lipid interactions in gluten elucidated using acetic acid fractionation.* Food Chem., 2009. **115**(1): p. 105-112.
120. Macritchie, F., *The role of lipids in baking*, in *Lipids in Cereal Technology*, P.J. Barnes, Editor. 1983, Academic Press: New York. p. 165-88.
121. Englyst, H.N., V. Anderson, and J.H. Cummings, *Starch and non-starch polysaccharides in some cereal foods.* J. Sci. Food Agric., 1983. **34**(12): p. 1434-40.
122. Sasaki, T., T. Yasui, and J. Matsuki, *Influence of non-starch polysaccharides isolated from wheat flour on the gelatinization and gelation of wheat starches.* Food Hydrocolloids, 2000. **14**(4): p. 295-303.
123. Rouau, X., M.L. El-Hayek, and D. Moreau, *Effect of an enzyme preparation containing pentosanases on the bread-making quality of flours in relation to changes in pentosan properties.* J. Cereal Sci., 1994. **19**(3): p. 259-72.
124. Wang, M., et al., *Interaction of Water Extractable Pentosans with Gluten Protein: Effect on Dough Properties and Gluten Quality.* J. Cereal Sci., 2002. **36**(1): p. 25-37.
125. Biliaderis, C.G., M.S. Izydorczyk, and O. Rattan, *Effect of arabinoxylans on bread-making quality of wheat flours.* Food Chem., 1995. **53**(2): p. 165-71.
126. Labat, E., X. Rouau, and M.-H. Morel, *Effect of flour water-extractable pentosans on molecular associations in gluten during mixing.* Lebensm.-Wiss. Technol., 2002. **35**(2): p. 185-189.
127. Wang, M., T. van Vliet, and R.J. Hamer, *Evidence that pentosans and xylanase affect the re-agglomeration of the gluten network.* J. Cereal Sci., 2004. **39**(3): p. 341-349.
128. Singh, N., H. Singh, and M.S. Bakshi, *Determining the distribution of ash in wheat using debranning and conductivity.* Food Chem., 1998. **62**(2): p. 169-172.

129. Peterson, C.J., V.A. Johnson, and P.J. Mattern, *Influence of cultivar and environment on mineral and protein concentrations of wheat flour, bran, and grain.* Cereal Chem., 1986. **63**(3): p. 183-6.
130. Pateras, I.M.C., *Bread Spoilage and Staling* in *Technology of Breadmaking*, S.P.C.a.L.S. Young, Editor. 2007, Springer US. p. 275-298.
131. Armero, E. and C. Collar, *Antistaling additive effects on fresh wheat bread quality.* Food Sci. Technol. Int. (London), 1996. **2**(5): p. 323-333.
132. Aamodt, A., E.M. Magnus, and E.M. Faergestad, *Effect of flour quality, ascorbic acid, and DATEM on dough rheological parameters and hearth loaves characteristics.* J. Food Sci., 2003. **68**(7): p. 2201-2210.
133. Skaf, A., et al., *A new acoustic technique to monitor bread dough during the fermentation phase* Journal of Food Engineering, 2009. **93**(3): p. 365-378.
134. Lin, H.-I., *Using enzymes to improve frozen-dough bread quality*, in *Department of Grain Science and Industry*. 2008, Kansas State University: Manhattan. p. 90.
135. Williams, T. and G. Pullen, *Functional Ingredients*, in *Technology of Breadmaking*. 2007. p. 51-91.
136. Charley, H., *Food Science. 2nd Ed*. 1982. 564 pp.
137. Stampfli, L. and B. Nersten, *Emulsifiers in bread making.* Food Chem., 1995. **52**(4): p. 353-60.
138. Addo, K. and Y. Pomeranz, *Effects of lipids and emulsifiers on alveograph characteristics.* Cereal Chem., 1992. **69**(1): p. 6-12.
139. Mandala, I., A. Polaki, and S. Yanniotis, *Influence of frozen storage on bread enriched with different ingredients.* Journal of Food Engineering, 2009. **92**(2): p. 137-145.
140. Linko, Y.-Y., P. Javanainen, and S. Linko, *Biotechnology of bread baking.* Trends in Food Science & Technology, 1997. **8**(10): p. 339-344.
141. *Enzyme Kinetics*, in *Fundamentals of Biochemical Engineering*. 2008. p. 8-49.
142. Arcot, J., A.K. Shrestha, and U. Gusanov, *Enzyme protein binding assay for determining folic acid in fortified cereal foods and stability of folic acid under different extraction conditions.* Food Control. **13**(4-5): p. 245-252.
143. *Enzymes*, in *Food Chem*. 2009. p. 93-157.
144. Poutanen, K., *Enzymes: An important tool in the improvement of the quality of cereal foods.* Trends in Food Science & Technology, 1997. **8**(9): p. 300-306.
145. Chaplin, M.F., C. Bucke, and Editors, *Enzyme Technology*. 1990. 264 pp.
146. Tucker, G.A., L.F.J. Woods, and Editors, *Enzymes in Food Processing*. 1995. 320 pp.
147. Whitehurst, R.J. and B.A. Law, *Enzymes in Food Technology*. 2002. 255 pp.
148. Wong, D. and Editor, *Food Enzymes: Structure and Mechanism*. 1995. 408 pp.
149. Reed, G. and Editor, *Food Science and Technology Series: Enzymes in Food Processing. 2nd Ed*. 1975. 573 pp.
150. Christophersen, C. and D.E.O.B.E.N.S.C.T. Schäfer, *Enzymatic Characterisation* Starch - Stärke, 1998. **50**(1): p. 39-45.
151. Muralikrishna, G. and M. Nirmala, *Cereal α-amylases - an overview.* Carbohydrate Polymers, 2005. **60**(2): p. 163-173.
152. Goesaert, H., et al., *Antifirming Effects of Starch Degrading Enzymes in Bread Crumb.* J. Agric. Food Chem, 2009. **57**(6): p. 2346-2355.
153. Palacios, H.R., P.B. Schwarz, and B.L. D'Appolonia, *Effect of α-Amylases from Different Sources on the Retrogradation and Recrystallization of Concentrated Wheat Starch Gels: Relationship to Bread Staling.* J. Agric. Food Chem, 2004. **52**(19): p. 5978-5986.
154. Leman, P., et al., *Maltogenic amylase has a non-typical impact on the molecular and rheological properties of starch.* Carbohydr. Polym., 2005. **62**(3): p. 205-213.
155. Jeffries, T.W., *Biochemistry and genetics of microbial xylanases.* Curr. Opin. Biotechnol., 1996. **7**(3): p. 337-342.
156. Eliasson, A.C., *Starch gelatinization in the presence of emulsifiers. A morphological study of wheat starch.* Starch/Staerke, 1985. **37**(12): p. 411-15.

157. Ghiasi, K., R.C. Hoseney, and E. Varriano-Marston, *Gelatinization of wheat starch. I. Excess-water systems.* Cereal Chem., 1982. **59**(2): p. 81-5.
158. Azizi, M.H. and G.V. Rao, *Effect of storage of surfactant gels on the bread making quality of wheat flour.* Food Chem., 2004. **89**(1): p. 133-138.
159. Kamel, B.S. and R. Hoover, *Production of bread using sodium stearoyl lactylate as a replacement for shortening.* Food Res. Int., 1992. **25**(4): p. 285-8.
160. Byler, D.M. and H. Susi, *Examination of the secondary structure of proteins by deconvolved FTIR spectra.* Biopolymers, 1986. **25**(3): p. 469-487.
161. Elliott A, A.E., *structure of synthetic polypeptides.* nature, 1950. **165**: p. 921-922.
162. Krimm S, B.J., *vibrational spectroscopy and conformation of peptides, polypeptides, and proteins.* advance protein chemistry, 1986. **38**: p. 181-364.
163. Kong, J. and S. Yu, *Fourier Transform Infrared Spectroscopic Analysis of Protein Secondary Structures.* Acta Biochimica et Biophysica Sinica, 2007. **39**(8): p. 549–559.
164. Hind, A.R., S.K. Bhargava, and A. McKinnon, *At the solid/liquid interface: FTIR/ATR the tool of choice.* Advances in Colloid and Interface Science, 2001. **93**(1-3): p. 91-114.
165. Fink, D.J. and R.M. Gendreau, *Quantitative surface studies of protein adsorption by infrared spectroscopy : I. Correction for bulk concentrations.* Analytical Biochem, 1984. **139**(1): p. 140-148.
166. Maréchal, Y., *Interaction configurations of H_2O molecules in a protein (Stratum Corneum) by infrared spectrometry* journal of molecular structure, 1997. **416**(1-3): p. 133-143.
167. Grdadolnik, J. and Y. Maréchal, *Bovine Serum Albumin Observed by Infrared Spectrometry.I. Methodology, Structural Investigation, and Water Uptake.* Biopolymers (Biospectroscopy), 2001. **62**: p. 40-53.
168. Grdadolnik, J. and Y. Maréchal, Biopolymers (Biospectroscopy), 2001. **62**: p. 40-53.
169. Krimm, S., J. Mol. Biol, 1962. **4**: p. 528–540.
170. Jackson, M. and C.H.H. Mantsch, J. Chem., 1991. **69**: p. 1639–1642.
171. Besset, F., J. Spectr, 1975. **20**: p. 126-136.
172. Arrondoa, J.L.R. and F.M. Goni, *Structure and dynamics of membrane proteins as studied by infrared spectroscopy.* Progress in Biophysics and Molecular Biology, 1999. **72**: p. 367–405.
173. Grdadolnik, J., *Saturation effects in FTIR spectroscopy: intensity of amide I and amide II bands in protein spectra.* Acta Chim. Slov., 2003. **50**(4): p. 777-788.
174. Surewicz, W.K. and H.H. Mantsch, *New insight into protein secondary structure from resolution-enhanced infrared spectra.* Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 1988. **952**: p. 115-130.
175. Jackson, M. and H.H. Mantsch, *Protein secondary structure from FT-IR spectroscopy: correlation with dihedral angles from three-dimensional Ramachandran plots.* Can. J. Chem., 1991. **69**(11): p. 1639-42.
176. Bandekar, J., *Amide modes and protein conformation.* Biochim Biophys Acta., 1992. **1120**(2): p. 123-43.
177. Grdadolnik, J. and Y. Marechal, *Infrared difference spectroscopy Part II. Spectral Decomposition.* Vibrational Spectroscopy, 2003. **31**(2): p. 289-294.
178. Lee, D.C., et al., *Determination of protein secondary structure using factor analysis of infrared spectra.* Biochemistry., 1990. **29**(39): p. 9185-93.
179. Dousseau, F. and M. Pezolet, *Determination of the secondary structure content of proteins in aqueous solutions from their amide I and amide II infrared bands. Comparison between classical and partial least-squares methods.* Biochemistry, 1990. **29**(37): p. 8771-9.
180. Susi, H. and D.M. Byler, *Fourier transform infrared study of proteins with parallel beta -chains.* Arch. Biochem. Biophys., 1987. **258**(2): p. 465-9.
181. Byaruhanga, Y.B., et al., *Alteration of kafirin and kafirin film structure by heating with microwave energy and tannin complexation.* J. Agric. Food Chem., 2006. **54**(12): p. 4198-4207.

182. Lefevre, T. and M. Subirade, *Structural and interaction properties of beta - lactoglobulin as studied by FTIR spectroscopy.* Int. J. Food Sci. Technol., 1999. **34**(5/6): p. 419-428.
183. Purcell, J.M. and H. Susi, *Solvent denaturation of proteins as observed by resolution-enhanced Fourier transform infrared spectroscopy.* J. Biochem. Biophys. Methods, 1984. **9**(3): p. 193-9.
184. Dong, A., P. Huang, and W.S. Caughey, *Protein secondary structures in water from second-derivative amide I infrared spectra.* Biochemistry, 1990. **29**(13): p. 3303-8.
185. Lin, S.-Y., et al., *Effect of ethanol or/and captopril on the secondary structure of human serum albumin before and after protein binding.* Eur. J. Pharm. Biopharm., 2004. **57**(3): p. 457-464.
186. Cui, F.Z., et al., *Irradiation effects on secondary structure of protein induced by keV ions.* Radiat. Phys. Chem., 2001. **60**(1-2): p. 35-38.
187. Robertson, G.H., K.S. Gregorski, and T.K. Cao, *Changes in secondary protein structures during mixing development of high absorption (90%) flour and water mixtures.* Cereal Chem., 2006. **83**(2): p. 136-142.
188. Popineau, Y., et al., *A study by infrared spectroscopy of the conformations of gluten proteins differing in their gliadin and glutenin compositions.* J. Cereal Sci., 1994. **20**(1): p. 15-22.
189. Mejri, M., et al., *Effects of some additives on wheat gluten solubility: A structural approach.* Food Chem., 2005. **92**(1): p. 7-15.
190. Servaty, R., et al., *Hydration of polymeric components of cartilage - an infrared spectroscopic study on hyaluronic acid and chondroitin sulfate.* Int. J. Biol. Macromol., 2001. **28**(2): p. 121-127.
191. Starzak, M. and M. Mathlouthi, *Cluster composition of liquid water derived from laser-Raman spectra and molecular simulation data.* Food Chem., 2003. **82**(1): p. 3-22.
192. Rueegg, M. and H. Haeni, *Infrared spectroscopy of the water vapor sorption process of caseins.* Biochim. Biophys. Acta, Protein Struct., 1975. **400**(1): p. 17-23.
193. Subirade, M., et al., *Molecular basis of film formation from a soybean protein: comparison between the conformation of glycinin in aqueous solution and in films.* Int. J. Biol. Macromol., 1998. **23**(4): p. 241-249.
194. Seabourn, B.W., F. Xie, and O.K. Chung, *Rapid determination of dough optimum mixing time for early generation wheat breeding lines using FT-HATR infrared spectroscopy.* Crop Sci., 2008. **48**(4): p. 1575-1578.
195. Georget, D.M.R. and P.S. Belton, *Effects of Temperature and Water Content on the Secondary Structure of Wheat Gluten Studied by FTIR Spectroscopy.* Biomacromolecules, 2006. **7**(2): p. 469-475.
196. Belton, P.S., et al., *FTIR and NMR studies on the hydration of a high-Mr subunit of glutenin.* Int. J. Biol. Macromol., 1995. **17**(2): p. 74-80.
197. Secundo, F. and N. Guerrieri, *ATR-FT/IR Study on the Interactions between Gliadins and Dextrin and Their Effects on Protein Secondary Structure.* J. Agric. Food Chem., 2005. **53**(5): p. 1757-1764.
198. Agrawal, M., et al., *Hydrolysis of starch by amylase from Bacillus sp. KCA102: a statistical approach.* Process Biochem. (Oxford, U. K.), 2005. **40**(7): p. 2499-2507.
199. Goodfellow, B.J. and R.H. Wilson, *A Fourier transform IR study of the gelation of amylose and amylopectin.* Biopolymers, 1990. **30**(13-14): p. 1183-9.
200. Kacurakova, M. and M. Mathlouthi, *FTIR and laser-Raman spectra of oligosaccharides in water: characterization of the glycosidic bond.* Carbohydr. Res., 1996. **284**(2): p. 145-157.
201. Schuster, K.C., et al., *On-line FT-Raman spectroscopic monitoring of starch gelatinisation and enzyme catalysed starch hydrolysis.* Vibrational Spectroscopy, 2000. **22**(1-2): p. 181-190.
202. Boussarsar, H., B. Rogé, and M. Mathlouthi, *Physico-chemical Approach of the Amylolytic Action Pattern of a Thermostable Amylopullulanase* Food Biophysics, 2007. **Volume 2**(2-3): p. 100-107.

203. Kacurakova, M. and R.H. Wilson, *Developments in mid-infrared FT-IR spectroscopy of selected carbohydrates.* Carbohydr. Polym., 2001. **44**(4): p. 291-303.
204. Sekkal, M., et al., *Investigation of the glycosidic linkages in several oligosaccharides using FT-IR and FT Raman spectroscopies.* J. Mol. Struct., 1995. **349**: p. 349-52.
205. Copikova, J., et al., *Influence of hydration of food additive polysaccharides on FT-IR spectra distinction.* Carbohydr. Polym., 2006. **63**(3): p. 355-359.
206. Sevenou, O., et al., *Organisation of the external region of the starch granule as determined by infrared spectroscopy.* Int J Biol Macromol, 2002. **31**(1-3): p. 79-85.
207. Hancock, R.D. and B.J. Tarbet, *The other double helix - the fascinating chemistry of starch.* J. Chem. Educ., 2000. **77**(8): p. 988-992.
208. Cael, J.J., J.L. Koenig, and J. Blackwell, *Infrared and Raman spectroscopy of carbohydrates. VI. Normal coordinate analysis of V-amylose.* Biopolymers, 1975. **14**(9): p. 1885-903.
209. Cael, J.J., et al., *Infrared and Raman spectroscopy of carbohydrates. V. Normal coordinate analysis of cellulose I.* J. Chem. Phys., 1975. **62**(3): p. 1145-53.
210. Cael, J.J., J.L. Koenig, and J. Blackwell, *Infrared and Raman spectroscopy of carbohydrates. IV. Identification of configuration-and conformation-sensitive modes for D-glucose by normal coordinate analysis.* Carbohyd. Res., 1974. **32**(1): p. 79-91.
211. Cael, J.J., J.L. Koenig, and J. Blackwell, *Infrared and Raman spectroscopy of carbohydrates. III. Raman spectra of the polymorphic forms of amylose.* Carbohyd. Res., 1973. **29**(1): p. 123-34.
212. Vasko, P.D., J. Blackwell, and J.L. Koenig, *Infrared and Raman Spectroscopy of carbohydrates. II. Normal coordinate analysis of alpha -D-glucose.* Carbohyd. Res., 1972. **23**(3): p. 407-16.
213. Xie, X., Q. Liu, and S.W. Cui, *Studies on the granular structure of resistant starches (type 4) from normal, high amylose and waxy corn starch citrates.* Food Res. Int., 2006. **39**(3): p. 332-341.
214. Wolkers, W.F., et al., *A Fourier-transform infrared spectroscopy study of sugar glasses.* Carbohydr. Res., 2004. **339**(6): p. 1077-1085.
215. van Soest, J.J.G., et al., *Short-range structure in (partially) crystalline potato starch determined with attenuated total reflectance Fourier-transform IR spectroscopy.* Carbohydr. Res., 1995. **279**: p. 201-14.
216. Rubens, P., et al., *In situ observation of pressure-induced gelation of starches studied with FTIR in the diamond anvil cell.* Carbohydr. Polym., 1999. **39**(3): p. 231-235.
217. Wilson, R.H. and P.S. Belton, *A Fourier-transform infrared study of wheat starch gels.* Carbohydr. Res., 1988. **180**(2): p. 339-44.
218. Smits, A.L.M., et al., *Aging of starch based systems as observed with FT-IR and solid state NMR spectroscopy.* Starch/Staerke, 1998. **50**(11-12): p. 478-483.
219. www.landfood.ubc.ca/.../301/water/waterq5.htm.
220. Susi, H. and D.M. Byler, *Protein structure by Fourier transform infrared spectroscopy: second derivative spectra.* Biochemical and Biophysical Research Communications, 1983. **115**(1): p. 391-7.
221. Dousseau, F., M. Therrien, and M. Pezolet, *On the spectral subtraction of water from the FT-IR spectra of aqueous solutions of proteins.* Applied Spectroscopy, 1989. **43**(3): p. 538-42.
222. Byler, D.M. and H. Susi, *Examination of the secondary structure of proteins by deconvolved FTIR spectra.* Biopolymers, 1986. **25**(3): p. 469-87.
223. Barilaro, D., et al., *FT-IR absorbance spectroscopy to study Sicilian "proto-majolica" pottery.* Vibrational Spectroscopy, 2008. **48**(2): p. 269-275.
224. Byler, D.M., J.N. Brouillette, and H. Susi, *Quantitative studies of protein structure by FT-IR spectral deconvolution and curve fitting.* Spectroscopy (Duluth, MN, United States), 1986. **1**(3): p. 29-32.
225. Farvili, N., C.E. Walker, and J. Qarooni, *Effects of Emulsifiers on Pita Bread Quality.* j. cereal sci, 1995. **21**(3): p. 301-308.

226. Gomez, M., et al., *Functionality of different emulsifiers on the performance of breadmaking and wheat bread quality.* Eur. Food Res. Technol., 2004. **219**(2): p. 145-150.
227. Iorga, E., et al., *Synergistic effect of bacterial and fungal amylases on breadmaking.* Roum. Biotechnol. Lett., 2003. **8**(1): p. 1129-1138.
228. Sanz Penella, J.M., C. Collar, and M. Haros, *Effect of wheat bran and enzyme addition on dough functional performance and phytic acid levels in bread.* J.Cereal Sci, 2008. **48**(3): p. 715-721.
229. Z.Gan, *Gas Cell stabilization and Gas Retention in Wheat Bread Dough.* journal of cereal science, 1994. **21**: p. 215-230.
230. Kim, Y.-R. and P. Corni, *Effects of temperature and mixing time on molecular mobility in wheat dough.* LWT - Food Science and Technology, 2001. **34**(7): p. 417-423.
231. K. Wehrle, H.G., E. K. Arendt and *Effects of Lactic Acid, Acetic Acid, and Table Salt on Fundamental Rheological Properties of Wheat Dough*
Cereal Chemistry, 1997. **74**(6): p. 739-744.
232. Hoseney, R.C., *Principals of Cereal Science and Technology.* St:paulAmerican Association of Cereal Chemists, 1986: p. 205-206.
233. Gujral, H.S.a.N.S., *Effect of additives on dough development, gaseous release and bread making properties.* Food Research International, 2000. **32**(10): p. 691-697.
234. Kim, Y.-R. and P. Cornillon, *Effects of temperature and mixing time on molecular mobility in wheat dough.* Lebensmittel-Wissenschaft und -Technologie, 2001. **34**(7): p. 417-423.
235. Seetharaman, K., N. Yao, and M.K. Rout, *Role of water in pretzel dough development and final product quality.* Cereal Chemistry, 2004. **81**((3)): p. 336-340.
236. R., O.J., *measurement of flour color in color space parameters.* Cereal Chemistry, 1992. **69**(5): p. 546-551.
237. Jooyandeh, H., African Journal of Food Science., 2009. **3**(2): p. 028-034.
238. Owen, R.F., *Food chemistry*, ed. r. ed. 1996, New York: Marcel Dekker.
239. Purlis, E. and V.O. Salvadori, *Bread browning kinetics during baking.* Journal of Food Engineering, 2007. **80**(4): p. 1107–1115.
240. Bollain, C., A. Angioloni, and C. Collar, *Relationships between dough and bread viscoelastic properties in enzyme supplemented wheat samples.* Journal of Food Engineering, 2006. **77**(3): p. 665-671.
241. Zanoni, B., C. Peri, and D. Bruno, *Modeling of browning kinetics of bread crust during baking.* Food Science & Technology (London), 1995. **28**(6): p. 604-9.
242. http://www.lallemand.com/BakerYeastNA/eng/PDFs/LBU%20PDF%20FILES/1_7REDUC.PDF. 2006 [cited 1.
243. HOSENEY, W.D.R.C., *Effect of Certain Bread Making Oxidants and Reducing Agents on Dough Rheological Properties.* Cereal Chem, 1995. **72**(1): p. 58-64.
244. Aminlari, M. and M. Majzoobi, *Effect ofChemical Modification, pH change, and Freezing on the Rheological,Solubility,and Electrophoretic Pattern of Wheat Flour Proteins.* Journal of Food Science, 2002. **67**(7): p. 2502-2506.
245. Koh, B.-K., G.-C. Lee, and S.-T. Lim, *Effect of Amino Acids and Peptides on Mixing and Frozen Dough Properties of Wheat flour.* journal of cereal science, 2005. **70**(6): p. 359-364.
246. Horvat, R.J., H. Ng, and J. Pence, *Amino acids in fermented bread doughs.* journal of cereal science, 1962. **27**: p. 583-586.
247. Hoseney, J.M.F.a.R.C., *the Viscoelastic properties of wheat flour dough*, in *Dough Rheology and Baked Product Texture*, H.A. Faridi, Editor. 1989, Chapman & Hall: New York. p. 29-66.
248. Ravi, R., R.S. Manohar, and P.H. Rao, *Influence of additives on the rheological characteristics and baking quality of wheat flours.* Eur. Food Res. Technol., 2000. **210**(3): p. 202-208.
249. Lambert, I.A. and J.L. Kokini, *Effect of L-cysteine on the rheological properties of wheat flour.* Cereal Chem., 2001. **78**(3): p. 226-230.

250. Jalaludin Qarooni, M.W., Graham McMaster, *Factors affecting the quality of Arabic bread - additional ingredients.* J Agric Food Chem, 1989. **48**(2): p. 235-244.
251. Hruskova, M. and D. Novotna, *Effect of ascorbic acid on the rheological properties of wheat fermented dough.* Czech J. Food Sci., 2003. **21**(4): p. 137-144.
252. Dong, W. and R.C. Hoseney, *Effects of certain breadmaking oxidants and reducing agents on dough rheological properties.* Cereal Chem., 1995. **72**(1): p. 58-64.
253. Rodriguez-Mateos, A., et al., *Formation of dityrosine cross-links during breadmaking.* J Agric Food Chem, 2006. **54**(7): p. 2761-6.
254. Grant, D.R., *Role of ascorbic acid in chemical dough development. I. Reaction of ascorbic acid with flour-water suspensions.* Cereal Chem., 1974. **51**(5): p. 684-92.
255. Suarez-Luque, S., et al., *Different forms of maleic and fumaric acids (cis and trans of 2-butenedioic acid) in honey.* Food Chem., 2002. **80**(2): p. 215-219.
256. Moresi, M., et al., *Optimization of fumaric acid production from potato flour by Rhizopus arrhizus.* Applied Microbiology and Biotechnology, 1991. **36**(1): p. 35-39.
257. Francis, J.J. and Editor, *Wiley Encyclopedia of Food Science and Technology, Second Edition, 4-Volume Set*. 1999. 3840 pp.
258. Schroeder, L.F. and R.C. Hoseney, *Mixograph studies. II. Effect of activated double-bond compounds on dough-mixing properties.* Cereal Chem., 1978. **55**(3): p. 348-60.
259. http://www.bartek.ca/pdfs/BulletinsFumaric/Bartek%20Fumaric%20Acid%20in%20Wheat%20and%20Corn%20Flour%20Tortillas.pdf.
260. http://www.bartek.ca/pdfs/BulletinsFumaric/Technical%20Bulletin%20Fumaric%20Acid%20in%20Wheat%20Flour%20Tortillas.pdf.
261. Matz, S.P.-T.I., USA), *ingredients from wheat*, in *Bakery Technology and Engineering*. March 1992, Kluwer Academic Publishers Group. p. 10.
262. Haros, M., C.M. Rosell, and C. Benedito, *Effect of different carbohydrases on fresh bread texture and bread staling.* Eur. Food Res. Technol., 2002. **215**(5): p. 425-430.
263. Curic, D., J. Dugum, and I. Bauman, *The influence of fungal alpha -amylase supplementation on amylolytic activity and baking quality of flour.* Int. J. Food Sci. Technol., 2002. **37**(6): p. 673-680.
264. Martinez-Anaya, M.A. and T. Jimenez, *Functionality of enzymes that hydrolyze starch and non-starch polysaccharide in breadmaking.* Z. Lebensm.-Unters. Forsch. A, 1997. **205**(3): p. 209-214.
265. Sievert, D., R.C. Hoseney, and J.A. Delcour, *Starch*, in *bread and other baked products*. 2007, Wiley-VCH Verlag GmbH & Co. KGaA. p. 7.
266. Doğan, İ.S., *Effect of α-amylases on dough properties during Turkish hearth bread production.* Int. J. Food Sci. Technol., 2003. **38**(2): p. 209-216.
267. Jòzef, S., *The Use of Starch Processing Enzymes in the Food Industry* in *Industrial Enzymes*, J.P.a.A.P. MacCabe, Editor. 2007, Springer Netherlands. p. 19-34.
268. Sievert, D., R.C. Hoseney, and J.A. Delcour, *Flavour of baked products*, in *bread and other baked products*. 2007, Wiley-VCH Verlag GmbH & Co. KGaA. p. 28.
269. Hassan Ahmadi Gavilighi, M.H.A., Mohsen Barzegar and Majid Arab Ameri, *Effect of Selected Hydrocolloids on Bread Staling as Evaluated by DSC and XRD.* Journal of Food Technology, 2006. **4**(3): p. 185-188.
270. Fiszman, S.M., A. Salvador, and P. Varela, *Methodological developments in bread staling assessment: application to enzyme-supplemented brown pan bread* Eur. Food Res. Technol., 2005. **221**(5): p. 613-623.
271. Eliasson, A.-C., M. Gudmundsson, and G. Svensson, *Thermal behaviour of wheat starch in flour -- Relation to flour quality.* LWT - Food Science and Technology, 1995. **28**(2): p. 227-235.
272. van der Maarel, M.J.E.C., et al., *Properties and applications of starch-converting enzymes of the [alpha]-amylase family.* Journal of Biotechnology, 2002. **94**(2): p. 137-155.

273. Mitsuiki, S., et al., *Comparative characterization of raw starch hydrolyzing [alpha]-amylases from various Bacillus strains.* Enzyme and Microbial Technology, 2005. **37**(4): p. 410-416.
274. Tester, R.F., T. Patel, and S.E. Harding, *Damaged starch characterisation by ultracentrifugation.* Carbohydrate Research, 2006. **341**(1): p. 130-137.
275. Miralbes, C., *Quality control in the milling industry using near infrared transmittance spectroscopy.* Food Chem, 2004. **88**(4): p. 621-628.
276. Mirabella, F.M., *Internal reflection spectroscopy : theory and applications* Practical spectroscopy series. 1993, New York Marcel Dekker. 374.
277. Sammon, C., et al., *The application of attenuated total reflectance Fourier transform infrared spectroscopy to monitor the concentration and state of water in solutions of a thermally responsive cellulose ether during gelation.* Polymer, 2006. **47**(2): p. 577-584.
278. Öhman, M., D. Persson, and C. Leygraf, *In situ ATR-FTIR studies of the aluminium/polymer interface upon exposure to water and electrolyte.* Progress in Organic Coatings, 2006. **57**(1): p. 78-88.
279. van Velzen, E.J.J., et al., *Factors associated with dough stickiness as sensed by attenuated total reflectance infrared spectroscopy.* Cereal Chem, 2003. **80**(4): p. 378-382.
280. Iizuka, K. and T. Aishima, *Starch gelation process observed by FT-IR/ATR spectrometry with multivariate data analysis.* J. Food Sci, 1999. **64**(4): p. 653-658.
281. Schindler, R., B. Lendl, and R. Kellner, *Simultaneous determination of [alpha]-amylase and amyloglucosidase activities using flow injection analysis with fourier transform infrared spectroscopic detection and partial least-squares data treatment.* Analytica Chimica Acta, 1998. **366**(1-3): p. 35-43.
282. Bellon-Maurel, V., C. Vallat, and D. Goffinet, *Quantitative analysis of individual sugars during starch hydrolysis by FT-IR/ATR spectrometry. Part II: Influence of external factors and wavelength parameters.* Applied Spectroscopy, 1995. **49**(5): p. 563-8.
283. Kapusniak, J. and P. Siemion, *Thermal reactions of starch with long-chain unsaturated fatty acids. Part 2. Linoleic acid.* Journal of Food Engineering, 2006. **78**(1): p. 323-332.
284. Mondal, K., A. Sharma, and M.N. Gupta, *Three phase partitioning of starch and its structural consequences.* Carbohydrate Polymers, 2004. **56**(3): p. 355-359.
285. van Staden, J.F. and L.V. Mulaudzi, *Flow injection spectrophotometric assay of a-amylase activity.* Analytica Chimica Acta, 2000. **421**(1): p. 19-25.
286. Athawale, V.D. and V. Lele, *Thermal studies on granular maize starch and its graft copolymers with vinyl monomers.* Starch/Staerke, 2000. **52**(6-7): p. 205-213.
287. Mejia, C.D., L.J. Mauer, and B.R. Hamaker, *Similarities and differences in secondary structure of viscoelastic polymers of maize a-zein and wheat gluten proteins.* J.Cereal Sci, 2007. **45**(3): p. 353-359.
288. Kizil, R., J. Irudayaraj, and K. Seetharaman, *Characterization of Irradiated Starches by Using FT-Raman and FTIR Spectroscopy.* J Agri Food Chem, 2002. **50**(14): p. 3912-3918.
289. Oneh Abu, J., K. Gyebi Duodu, and A. Minnaar, *Effect of g-irradiation on some physicochemical and thermal properties of cowpea (Vigna unguiculata L. Walp) starch.* Food Chem, 2005. **95**(3): p. 386-393.
290. Azevedo, H.S., F.M. Gama, and R.L. Reis, *In Vitro Assessment of the Enzymatic Degradation of Several Starch Based Biomaterials.* Biomacromolecules, 2003. **4**(6): p. 1703-1712.
291. Vasques, C.T., et al., *Effect of thermal treatment on the stability and structure of maize starch cast films.* Starch/Staerke, 2007. **59**(3-4): p. 161-170.
292. Krieg, P., et al., *Determination of a-amylase activity using Fourier-transform IR spectroscopy.* Fresenius' Journal of Analytical Chemistry, 1996. **356**(8): p. 504-507.
293. Khalid, E.K., E.E. Babiker, and A.H. El Tinay, *Solubility and functional properties of sesame seed proteins as influenced by pH and/or salt concentration.* Food Chem, 2003. **82**(3): p. 361-366.

294. Popineau, Y., et al., *Foaming and Emulsifying Properties of Fractions of Gluten Peptides Obtained by Limited Enzymatic Hydrolysis and Ultrafiltration.* J. Cereal Sci, 2002. **35**(3): p. 327-335.
295. Campos, D.T., J.F. Steffe, and P.K.W. Ng, *Mixing wheat flour and ice to form undeveloped dough.* Cereal Chem, 1996. **73**(1): p. 105-7.
296. Schluentz, E.J., J.F. Steffe, and P.K.W. Ng, *Rheology and microstructure of wheat dough developed with controled deformation.* Journal of Texture Studies, 2000. **31**(1): p. 41-54.
297. Agyare, K.K., et al., *Dynamic rheological and thermal properties of soft wheat flour dough containing structured lipid.* J. Food Sci, 2004. **69**(7): p. E297-E302.
298. Angioloni, A. and M. Dalla Rosa, *Effects of cysteine and mixing conditions on white/whole dough rheological properties.* Journal of Food Engineering, 2006. **80**(1): p. 18-23.
299. Chamberlain, N., *The use of ascorbic acid in breadmaking.* Vitam. C (Ascorbic Acid), 1981: p. 87-104.
300. Lee, J.W. and C.W. Wrigley, *The protein composition of gluten extracted from different wheats.* Australian J. Exptl. Agr. Animal Husbandry, 1963. **3**(9): p. 85-8.
301. Singh, H. and F. MacRitchie, *Use of sonication to probe wheat gluten structure.* Cereal Chem, 2001. **78**(5): p. 526-529.
302. Engelsen, S.B., et al., *NMR-baking and Multivariate Prediction of Instrumental Texture Parameters in Bread.* J. Cereal Sci, 2001. **33**(1): p. 59-69.
303. Haris, P.I., *Fourier transform infrared spectroscopic studies of peptides: potentials and pitfalls.* ACS Symposium Series, 2000. **750**(Infrared Analysis of Peptides and Proteins): p. 54-95.
304. Hering, J.A., P.R. Innocent, and P.I. Haris, *Automatic amide I frequency selection for rapid quantification of protein secondary structure from Fourier transform infrared spectra of proteins.* Proteomics, 2002. **2**(7): p. 839-849.
305. Yang, J.T., *Quantitative IR spectrophotometry of peptide compounds in water (H2O) solutions: Spectral parameters of amino acid residue absorption bands. Amide absorption bands of polypeptides and fibrous proteins in a-, b-, and random coil conformations. Estimation of the protein secondary structure.* Chemtracts: Biochemistry and Molecular Biology, 1991. **2**(4): p. 239-44.
306. Li-Chan, E.C.Y., et al., *Vibrational Spectroscopy of Food and Food Products.* John Wiley & Sons Ltd, 2002.
307. Nevskaya, C., Biopolymers, 1976. **4**: p. 637-48.
308. Nakanishi, M. and M. Tsuboi, *Determination of the number of hydrogen bonds in a protein molecule.* Bulletin of the Chemical Society of Japan, 1974. **47**(2): p. 305-7.
309. Arrondo, J.L.R., et al., *Quantitative studies of the structure of proteins in solution by Fourier-transform infrared spectroscopy.* Progress in Biophysics & Molecular Biology, 1993. **59**(1): p. 23-56.
310. Bruun Susanne, W., et al., *Correcting attenuated total reflection-Fourier transform infrared spectra for water vapor and carbon dioxide.* Appl Spectrosc, 2006. **60**(9): p. 1029-39.
311. Carbonaro, M., et al., *Application of Fourier transform infrared spectroscopy to legume seed flour analysis.* Food Chem, 2008. **108**(1): p. 361-368.
312. Schofield, J.D. and X. Chen, *Analysis of free reduced and free oxidised glutathione in wheat flour.* J. Cereal Sci, 1995. **21**(2): p. 127-136.
313. Jones, I.K. and P.R. Carnegie, *Rheological activity of peptides, simple disulphides and simple thiols in wheaten dough.* J.Agri Food Chem, 1969. **20**(1): p. 60-64.
314. Ewart, J.A.D., *Thiols in flour and baking quality.* Food Chem, 1990. **38**(1): p. 41-60.
315. Jones, I.K. and P.R. Carnegie, *Binding of oxidised glutathione to dough proteins and a new explanation, involving thiol disulphide exchange, of the physical properties of dough.* J.Agri Food Chem, 1971. **22**(7): p. 358-364.
316. Zentner, H., *Effect of ascorbic acid on wheat gluten.* J. Agri Food Chem, 1968. **19**(8): p. 464-467.

317. Koehler, P., *Effect of Ascorbic Acid in Dough: Reaction of Oxidized Glutathione with Reactive Thiol Groups of Wheat Glutelin.* J.Agri Food Chem, 2003. **51**(17): p. 4954-4959.
318. Bresler, S.E., *Structure, molecular forces and aggregation reactions of macromolecules of complex polymers.* Discuss. Faraday Soc., 1958. **No. 25**: p. 158-66.